STUDIES ON SOME LEAF SURFACE MICRO-ORGANISMS WITH SPECIAL REFERENCE TO THE PHYLLOPLANE OF ANTIRRHINUM MAJUS L.

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SUMMARY

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1) A survey has been made of the relevant literature on the colonisation of leaf surfaces by saprophytic microbes, the physical and chemical environment at the leaf surface and the interactions which may occur between saprophytic and parasitic microbes in the phylloplane.

2) Investigations into the saprophytic phylloplane microflora occurring on Antirrhinum majus L. Nanum and A. majus Fi hybrid tall forcing strain orange scarlet in field conditions were done during the 1972 growing season.

3) Filamentous fungi, yeasts and bacteria were isolated, in progressively increasing numbers during the season, from both cultivars.

4) The effects on the saprophytic phylloplane population following the artificial inoculation of antirrhinum plants in the field with the fungal rust pathogen Puccinia antirrhini D. & H. were investigated.

5) A marked increase in the numbers of the pink yeast Sporobolomyces roseus Kluyver & van Niel was observed on the leaves of the rust susceptible A. majus Nanum cultivar.

6) The major components of the saprophytic phylloplane microbial populations of both cultivars of antirrhinum were the pink yeast S. roseus and the dark pigmented filamentous fungus Cladosporium cladosporioides (Fresen.) de Vries.

7) The two saprophytic microbes S. roseus and C. cladosporioides together with the fungal rust pathogen P. antirrhini were selected for further experiments on the colonisation of leaves by saprophytes and the interactions, on the leaf surface, of these microbes with P. antirrhini.

8) A series of experiments was done to determine the effects of several physiological parameters on the germination and growth of the three microbes mentioned above.

9) All three microbes had an optimum temperature for germination and growth which was very sharply defined for P. antirrhini. The spore germination of both filamentous fungi required high humidities (100% R.H.) or the presence of free water. P. antirrhini germination and growth was inhibited by light but unaffected by the presence of exogenous carbohydrates in solution whereas both of the saprophytes were unaffected by light, but the germination and/or growth of both these microbes was stimulated in the presence of carbohydrates.

10) Leaf surfaces of both cultivars were shown, by scanning electron microscope studies, to be similar in that, although the cuticle was striated, almost no wax was observed on either side of the leaves. Stomatal numbers were higher on the abaxial leaf surface but no difference in numbers was observed between leaves of the same age of the two cultivars.

11) Leaf growth of both antirrhinum cultivars was measured over a five week period to enable Leaf Plastochron Indices to be established so that the physiologic age of all material used in experiments could be standardised.

12) Leaf leachates were found to contain both amino acids and carbohydrates, larger quantities of which were obtained from older leaves (Leaf Plastochron Index 1.5) of both cultivars. Also the leachates from the older leaves of A. majus Nanum contained about 11 times more carbohydrate than leachates from A. majus Fi hybrid leaves of the same physiologic age. The carbohydrates and amino acids found in leaf leachates of both cultivars were identical save that A. majus Fi hybrid leachates contained proline and hydroxyproline in addition to the other amino acids.

13) The aseptic culture of whole antirrhinum plants was attempted and eventually a workable system was found which only requires minor refinements before it may be used in studies on the phylloplane.

14) Leaf leachates of both antirrhinum cultivars inhibited germination and germ tube growth of P. antirrhini uredospores. A. majus Nanum leachates reduced germination by 20% and A. majus Fi hybrid leachates led to a 90% reduction in germination. However both saprophytic microbes were stimulated in the presence of leaf leachates from both cultivars.

15) There was variable colonisation by S. roseus and C. cladosporioides of attached antirrhinum leaves of both cultivars. However the total volume of cells of S. roseus did increase and the spore germination of C. cladosporioides increased rapidly to give a peak germination after four days before decreasing on the leaf midrib, no such pattern being observed on the leaf lamina. The growth of S. roseus and the germination and germ tube growth of

C. cladosporioides was reduced when either of these microbes was incubated with the other.

16) Colonisation of surface sterilised rooted detached leaves (on which results were less variable) by S. roseus showed that the increase in the total volume of yeast cells was affected by leaf age, plant cultivar and the presence of C. cladosporioides. Older leaves supported more growth than younger leaves and the older leaves of A. majus Nanum supported the greatest growth. The presence of C. cladosporioides significantly decreased the growth of the yeast on older leaves, but little effect was observed on younger leaves.

17) C. cladosporioides spore germination was more rapid and reached higher levels on the leaf midrib than on the lamina of detached antirrhinum leaves of both cultivars. Spore germination on the midrib was characterised by a peak value on day four on A. majus Nanum and on day five on A. majus F1 hybrid followed by a decrease which was thought to be associated with the formation of secondary spores leading to a lowering of the overall percentage germination. The presence of S. roseus delayed the time at which peak germination occurred by two days and also reduced the growth of spore germ tubes. On younger leaves spore germination was rapid during the first day after inoculation after which almost no further increase in germination occurred. Mean germ tube length increased initially and then only increased further after seven days incubation.

18) Spore germination and germ tube growth of P. antirrhini in vitro were inhibited by S. roseus but enhanced by the presence of

C. cladosporioides. Also growth of S. roseus was stimulated in the presence of P. antirrhini whereas C. cladosporioides was inhibited by either P. antirrhini or S. roseus.

19) Interactions between P. antirrhini, C. cladosporioides and S. roseus on the surface of leaves presented a complex picture. Under optimal conditions for P. antirrhini (predicted by in vitro tests) spore germination and germ tube growth were significantly reduced on A. majus Fi hybrid relative to A. majus Nanum. The presence of S. roseus enhanced spore germination of P. antirrhini on A. majus Fi hybrid, whereas C. cladosporioides reduced germination of A. majus Nanum leaves. S. roseus and C. cladosporioides were affected in the same way as in the in vitro tests excepting that on leaves S. roseus was inhibited by C. cladosporioides.

20) Under more natural conditions of differing night and day temperatures C. cladosporioides reduced germ tube growth and leaf penetration by P. antirrhini on both antirrhinum cultivars (no leaf penetration at all being observed on A. majus Fi hybrid leaves) and also reduced rust spore germination on A. majus Fi hybrid leaves. However, S. roseus enhanced P. antirrhini germ tube growth on A. majus Nanum but decreased germ tube growth on A. majus Fi hybrid and leaf penetration on A. majus Nanum. C. cladosporioides and S. roseus were affected in same manner as in the other experiment on detached leaves mentioned above.

21) Investigations into the saprophytic phylloplane microflora of Picea abies Karst. growing in Castle O'er Forest, Dumfriesshire in relation to infection of the trees by Chrysomyxa abietis Unger

were done in 1971. Numbers of filamentous fungi, yeasts and bacteria increased rapidly at the time of flushing of new needles and subsequently did not increase further. No consistent differences in numbers of microbes or changes in species composition of the saprophytic phylloplane microflora were observed on needles infected with C. abietis during the early stages of infection.

22) The release of sporidia of C. abietis was observed using a volumetric spore trap. The seasonal release appeared to be synchronised to the time of new needle flushing and a clear diurnal pattern of sporidia release at night was observed.

23) Investigations into the saprophytic phylloplane microflora of Acer pseudoplatanus L. growing at Newbattle Abbey, Midlothian, showed that numbers of filamentous fungi, yeasts and bacteria increased steadily throughout the growing season in 1971. An increase in the numbers of microbes, especially the pink yeast S. roseus, occurred when leaves became infected by the leaf pathogen Rhytisma acerinum (Persoon ex St. Amans) Fries.

24) Owing to thelack of success in germination and infection experiments with both C. abietis on P. abies and R. acerinum on A. pseudoplatanus, neither of these host/pathogen systems were selected for further detailed study.

25) An attempt has been made in discussion to relate some of these results with those obtained by other workers.

INTRODUCTION

INTRODUCTION

Our knowledge of the existence of micro-organisms which inhabit the aerial parts of plants dates from the pioneer work of Pasteur (1876) who studied the distribution of yeasts on the surfaces of fruits. Little further interest was shown in this habitat for micro-organisms until the 1950's. Last (1955a) and Ruinen (1956) investigated the saprophytic microflora of plant leaves and simultaneously adopted the term phyllosphere to describe this habitat. However, if direct synonymy is to be preserved with terms used in studies on plant roots, then the term phylloplane should be used when referring to the actual leaf surface (Kerling, 1958). Since this time many studies have been conducted into the saprophytic phylloplane microflora of many plant species. Such work has been reviewed extensively (Last & Deighton, 1965; Leben, 1965a; Sinha, 1965; Hudson, 1968; Last & Warren, 1972). Many plant species have been studied but little work appears to have been done on evergreen perennial species, although the saprophytic mycoflora of Eucalyptus regnans F. Muell. has been described by Macauley & Thrower (1966).

Most of these studies have related the overall change in microbial numbers to season and to leaf age although few authors have distinguished between the effects of these two factors. The saprophytic phylloplane microflora might be expected to be affected by the nutrients available on the leaf surface (Tukey, 1971) and the presence of inhibitory substances such as phytoalexins (Cruickshank, 1962). The qualitative and quantitative composition of the saprophytic phylloplane microflora has been shown to depend on plant species (Kerling, 1964), leaf age (Last, 1955a; Dickinson, 1967), and

environmental conditions (Leben, 1965b; Jensen, 1971). Also the treatment of the leaf by fungicides (Hislop & Cox, 1969) or other chemicals (Crosse et al. 1968) may affect the saprophytes on the phylloplane.

There have been doubts about the precise activity on the leaf surface of some of the saprophytic micro-organisms isolated from leaves. Leben (1965a) stated that it was possible to distinguish 'casual' and 'resident' micro-organisms in the phylloplane microflora. The former remain inactive or develop only on organic debris deposited on the leaf from elsewhere, while the latter group grow actively on the leaf surface.

The inability of some organisms to actively colonise the leaf surface may be a major cause of the failure of saprophytic micro-organisms to achieve the biological control of pathogens predicted by Wood and Tveit (1955), as this failure has been ascribed to the inability of the antagonistic saprophytes to maintain themselves at high numbers (Goodman, 1967) or to withstand the environmental conditions experienced in the field (Leben et al. 1965). In view of this, it is surprising that few detailed studies of leaf surface colonisation by saprophytes have been done. However, the colonisation of sycamore leaves in the field has been observed (Pugh & Buckley, 1971a). In laboratory experiments on the colonisation of tomatoes (Leben, 1963), bean plants (Leben et al. 1970), larch seedlings (McBride, 1970) and soybeans (Mew & Kennedy, 1971; Scherff, 1973) the inoculation with cultures of single micro-organisms gave very variable results for the increase in numbers of the particular micro-organism. In each case this variability was ascribed to the effect of the environmental conditions surrounding the plants.

The study of micro-organisms on plant leaves under controlled environmental conditions has used techniques using small parts of plants such as leaf discs (Blakeman & Dickinson, 1967) and detached leaves (Malcolmson, 1969). However, observations obtained from such experiments are somewhat unsatisfactory as, although they give an indication of the effect of the factor tested, this may not represent the situation occurring on actively growing leaves as the physiological state of such excised plant material has been questioned (Cram, 1972).

The original aim of this project was to elucidate further the observations of Bier (1965) and McBride (1969, 1970, 1971) since both of these workers had shown the possibilities of control of pathogenic disease organisms using saprophytic micro-organisms isolated from the phylloplane. Crosse (1965) and Goodman (1965, 1967) had previously shown that antagonism of pathogens by saprophytes does occur on the leaves of tree species. Since few investigations have been carried out on perennial species, especially Gymnosperms, it seemed desirable to extend McBride's work to other conifers, provided that a suitable host/pathogen system could be found, particularly in view of the importance of conifers in British forestry. Proof of a regulating system here might have important implications in the possible biological control of certain tree diseases. Initially Picea abies (L.) Karst. was chosen as it had been planted widely and a specific needle disease caused by Chrysomyxa abietis Unger had been observed in several Scottish forests. At the same time Acer pseudoplatanus L. was studied, for although a detailed account of the phylloplane mycoflora of this tree had been given (Pugh & Buckley, 1971a) the effects of this microflora on the common leaf pathogen Rhytisma

acerinum (Pers. ex St. Amans) Fries were not known. Neither of these pathogens proved amenable to laboratory experimentation and the long incubation period of the pathogens precluded their use in a model system to study interactions on the phylloplane. The work on these species is included here as a record of the saprophytic microflora of each species and the effects on this microflora resulting from leaf infection by the relevant pathogen mentioned above.

After 15 months work on the perennial species, a search was made for another host/pathogen system which would be amenable to laboratory study. It was decided to use Antirrhinum majus L. with a specific pathogen Puccinia antirrhini D. & H., as the plants could be grown in the greenhouse throughout the year and the pathogen could be maintained on the leaves of such plants and required only a short incubation period.

The direction of this study was changed slightly to include the investigation of the saprophytic phylloplane microflora of antirrhinums and to use micro-organisms from this population as components in a model system set up to study in detail the initial colonisation of leaves by saprophytic micro-organisms, and the interactions of these micro-organisms with the obligate pathogen P. antirrhini on the leaf surface under closely controlled environmental conditions, and so the bulk of the work presented here is concerned with P. antirrhini on A. majus. Details of a system specially designed to culture antirrhinum plants under aseptic conditions are also reported.

PART I

ECOLOGY OF THE PHYLLOPLANE OF ANTIRRHINUM MAJUS NANUM AND ANTIRRHINUM MAJUS F1 HYBRID TALL FORCING STRAIN ORANGE SCARLET

SECTION I THE MICROBIAL COMPONENT

CHAPTER I SURVEY OF THE PHYLLOPLANE MICROFLORA OF A. MAJUS

INTRODUCTION

Antirrhinum majus L. (Plate 1) and its obligate rust pathogen Puccinia antirrhini D. & H. (Plate 2) were selected to form a suitable host/pathogen system for use in the study of microbial and plant/microbe interactions on leaf surfaces, both components being amenable to laboratory culture.

As only one reference to the isolation of saprophytes from leaves of antirrhinum was found in the literature (Last, 1970), it was necessary to survey their occurrence during the growing season. The most abundant microbes were selected for extended studies of leaf surface colonisation and for microbial interactions which might occur in this milieu, as it has been suggested that the ability of microbes to colonise leaves and persist in high numbers affects their potential to interact with plant pathogens (Newhook, 1957; Goodman, 1965; Crosse, 1965).

The effects on this microflora due to P. antirrhini inoculation of host plants in the field were also investigated.

Plate 1. *Antirrhinum majus* Nanum seedling.

Plate 2. Uredosori of *Puccinia antirrhini* on the lower surface of *A. majus* Nanum leaf

REVIEW OF LITERATURE

(a) SAPROPHYTIC MICROFLORA OF LEAVES

Although parasitic fungi attacking leaves and fruits of higher plants have attracted widespread interest, very few workers have investigated the non-parasitic microbes occurring on the aerial surfaces of plants. In the late nineteenth century Pasteur (1876, 1878) examined the distribution of yeast cells on the surfaces of fruits and suggested that numbers of microbes were related to the physiological changes occurring as fruits ripened. Since that time most studies of plant saprophytes have been centred on the microflora on root surfaces. However, during the 1950's interest in the study of microbes colonising the aerial parts of plants was awakened, this time with those colonising leaves. This interest was linked with adoption of the term 'phyllosphere' describing the external surface of the leaf as a milieu for microbes (Last, 1955a; Ruinen, 1956). Nevertheless, if direct synonomy with terms used in root investigations was to be retained, the term 'phyllosphere' should be widened to include the zone surrounding leaves and 'phylloplane' restricted to the actual leaf surfaces (Kerling, 1958). More recently the terms 'palynoplane' and 'palynosphere' have been proposed for the areas on and around pollen grains on the leaf surface (Diem, 1973). In recent years a great deal of research has been done on the phylloplane as an environment for microbes and also on the microbes themselves. As this field of interest has been reviewed on several occasions (Last & Deighton, 1965; Sinha, 1965; Leben, 1965a; Hudson, 1968; McBride, 1970; Last & Warren, 1972), the present survey will be restricted to

papers published during the last few years including the account of the Symposium on the Ecology of Leaf Surface Micro-Organisms held in Newcastle upon Tyne U.K. in 1970 (edit. Preece & Dickinson, 1971).

Recent papers by many groups of researchers have shown that there has been a concentration of interest in certain aspects of the phylloplane. The majority of these projects have used some form of cultural method for studying the phylloplane microflora. These methods have been reviewed by Dickinson (1971). A good example of a multimethod approach was the work of Pugh and Buckley (1971a) in which they used at least six different methods in the assessment of the phylloplane microflora.

One aspect of phylloplane research which has received attention recently concerns the possible sources of the epiphytic microflora found on leaf surfaces. The seed as a source of inoculum was examined by Leben (1965b), who found that bacteria migrated from seed to stem and leaves only when the relative humidity exceeded 90%. Commonly used seed dressing techniques decreased the numbers of saprophytes on the phylloplane (Klincare et al., 1971).

Buds provide another source of inoculum for perennial plants. Micro-organisms have been isolated from a variety of tree buds by several workers (Keener, 1950, 1951; Hislop & Cox, 1969; Pugh & Buckley, 1971b; Leben, 1972; Warren, personal communication). The numbers of organisms isolated from surface sterilised buds in the process of opening were greater than from dormant buds (Keener, 1950). Further, the outer bud scales from several tree species were found to be contaminated more frequently than inner bud scales (Pugh & Buckley, 1971b; Warren, personal communication). Keener (1951) was able to

isolate some micro-organisms from surface sterilised stem sections indicating that some phylloplane organisms may also live endophytically. This endophytic mode of existence was subsequently demonstrated for Aureobasidium pullulans (De Berg.) Arnaud (Pugh & Buckley, 1971b). When reviewing the epiphytic microflora of buds, Leben (1971) suggested that "this microflora might be manipulated in such a way as to prevent the establishment of some pathogenic bacteria, with a bud resident phase, from reaching the seed, thereby producing disease-free seed to be used for foundation stocks. This may be possible by growing plants in a greenhouse, where if the relative humidity is low and the buds are not wetted, numbers of bacterial residents in the bud are greatly reduced and so the pathogen would not be expected to be transferred from infected seed, via the bud, to the next seed generation" (Leben, 1971).

The deposition or impaction of airborne microbial particles is likely to be of importance in the dynamics of leaf surface colonisation by microbes. The spore content of the atmosphere has been studied (Hirst, 1953) and the spore trapping properties of leaves described (Gregory, 1971). The arrival of a fungal spore does not necessarily mean that this inoculation has been successful as the germination of the spore may be inhibited by the surface waxes on the leaf (Blakeman & Sztejnberg, 1973).

Recently the physical environment surrounding leaves and its effect on saprophytic microbes have received increasing attention. The literature on the environment at the surface of leaves has been reviewed by Burrage (1971). Some observed variations in the phylloplane microflora have been attributed to the changing physical conditions of the prevailing environment (di Menna, 1959). The

reduction of bacterial numbers on leaves after rain was thought to be attributable to the washing action of rain (Ruinen, 1961; McBride, 1970). On the other hand, Jensen (1971) observed that numbers of bacteria isolated from beech leaves dropped markedly during dry weather. It may well be that bacteria are particularly sensitive to changes in environmental conditions, although conditions of low humidity have been observed to cause a significant decline in the survival of growing fungi, especially the hyaline forms (Diem, 1971). Di Menna (1971) suggested that although climate affected phylloplane microbes, some of the changes observed might be due to seasonal effects. An overall rise in the numbers of micro-organisms during the growing season has been recorded by many workers (Last, 1955a; Kerling, 1958, 1964; Pugh, 1958; Hudson, 1962; Pugh & Buckley, 1971a). An investigation of the species of micro-organisms which occur on leaf surfaces during changing meteorological conditions at various periods during plant growth produced some interesting conclusions as to the relative importance of these factors on the development of the phylloplane microflora. Atmospheric humidity, temperature and rainfall are important factors influencing total numbers of micro-organisms and the specific composition of the microflora. The maturity of the leaf was found to be another major factor influencing the composition of the microflora. Invariably numbers of organisms increased with increasing leaf age (Sinha, 1971). The gradually increasing numbers of fungi may reflect the increasing deposition from the airspora over a long period, or may be due to the multiplication of micro-organisms in the phylloplane of older leaves observed by several workers (Last, 1955a; Ruinen, 1956; Stout, 1960; Kerling, 1964). The larger amounts of nutrients leached from older leaves might allow increasing colonisation by saprophytes (Tukey, 1971). Apart from this increase

in leachable material, the nutrient status of the leaf surface may be increased by the deposition of pollen. The presence of pollen stimulated a marked increase in numbers of saprophytic micro-organisms on the leaf surface (Fokkema, 1968; Warren, 1972a). Moreover, the infection of leaves by certain pathogens has been shown to stimulate an increase in the numbers of saprophytic yeasts on the phylloplane (Last, 1970). Thus it would appear that the interactions of many conditions control numbers of saprophytic micro-organisms on the phylloplane.

Recently research interest has increasingly concentrated on the effects of certain treatments used to control plant disease on saprophytic phylloplane micro-organisms. Applying the fungicide captan caused a marked reduction in numbers of viable saprophytic fungi isolated from leaves, but these numbers returned to the levels isolated from control plants within a few months (Hislop & Cox, 1969; Stott, 1971; Bainbridge & Dickinson, 1972; Warren, 1974). Bainbridge and Dickinson (1972) observed that captafol (structurally similar to captan) appeared to have no effect on bacterial numbers; neither did maneb or a mixture of maneb plus fentin acetate, but this latter fungicide appeared to affect A. pullulans and yeasts. Because of the lasting effects and the ease of application systemic fungicides are being increasingly applied to seeds. The effects of some of these compounds on the saprophytic phylloplane microflora have been investigated and benomyl and thio-bendazole have been shown to be extremely fungitoxic to some groups, especially Sporobolomyces roseus Kluyver & van Niel and A. pullulans (McKenzie, 1971; diMenna & Parle, 1972; Gross & Kenneth, 1973; Warren, 1974). This selectivity in supposedly wide spectrum fungicides completely changed the balance of

the saprophytic organisms in favour of white yeasts (Warren, 1974). This may have important consequences for the possibilities of the control of pathogens by major groups of saprophytic micro-organisms in the phylloplane observed by some workers (Fokkema, 1971; Warren, 1972b). As Furtado (1969) observed an increase in Coffee berry disease after plants had been sprayed with fungicides for some years, which he attributed to the resulting change in the saprophytic population on the sprayed leaves. A specific fungicide (ethirimol) has been shown to give good control of mildew without obviously affecting other phylloplane fungi or bacteria. It would be preferable to use compounds possessing similar specific activities towards pathogens though showing no adverse effects on the saprophytic phylloplane microflora which may exert some measure of disease control (Dickinson, 1973).

In these investigations the host plant itself has not been neglected. Some effects on the plant itself attributable to the saprophytes have been observed. Indole 3 acetic acid has been shown to be produced by several important groups of phylloplane micro-organisms including Cladosporium spp. (Valadon & Lodge, 1970), Epicoccum sp. and A. pullulans (Buckley & Pugh, 1971), S. roseus and Candida spp. (Diem & Hiem, 1971). Saprophytic organisms might also trigger phytoalexin production which might inhibit pathogen spore germination (Bailey, 1971). The enhancement of germination and of some agricultural plants has been ascribed to the presence of bacteria (Klincare et al., 1971). On the other hand, saprophytes may affect their host plants deleteriously. The premature senescence of leaves may be caused by some saprophytes (Skidmore & Dickinson, 1973). Furthermore, the pink yeast S. roseus has been shown to utilise leaf

waxes which might lead to a change in the permeability of the leaf surface (McBride, 1972).

(b) THE SAPROPHYTIC MICROFLORA OF ANTIRRHINUM MAJUS

Little is known about the saprophytic microflora on the leaves of A. majus, although S. roseus has been isolated from these leaves and would appear to be present in high numbers on leaves infected by the rust Puccinia antirrhini (Last, 1970).

(c) PUCCINIA ANTIRRHINI ON ANTIRRHINUM MAJUS

P. antirrhini, the cause of antirrhinum rust, is one of the few plant pathogens whose area of origin has been identified and whose spread around the world has been documented. Although A. majus is native in the Mediterranean region and the south west U.S.A., the rust was found on native antirrhinums only in California. The earliest record of this rust on cultivated antirrhinums was made in 1879 by Anderson in the area of Santa Cruz and in 1892 by Jepson (in Barbe, 1964) who observed it on the wild snapdragon Antirrhinum virga Gray. In 1895 Blasdale sent specimens of rusted antirrhinum to Dietel in Germany. Dietel recognised the pathogen as a new species and published the description of Puccinia antirrhini Dietel and Holway (Dietel, 1897). Only the uredospore and teleutospore stages of this rust have been found and although it is considered to be heteroecious alternate hosts have not been suggested. Antirrhinum infections may be maintained indefinitely by the uredospore stage (Wilson & Henderson, 1966). The rust has been cultivated successfully on several other antirrhinum hosts, but in nature it has only been collected from

Table 1 The first records of Puccinia antirrhini in many different countries

Date	Country or state	Reference
1901	U.S.A. Oregon	Barbe, 1964
1912	U.S.A. Illinois	"
1922	U.S.A. Texas, Florida	"
	Bermuda	"
	Canada Manitoba	"
1931	France	Lepik 1941
1933	England	Green 1933
1934	Germany	Andres 1935
	Denmark	Lepik 1941
1935	Sweden	Buchenwald 1936
	Italy; Switzerland	Lepik 1941
	Hungary; Austria	"
1936	Morócco; Egypt;	"
	Poland	"
1937	U.S.S.R.	"
1939	South Africa	Bottomley 1940
1941	U.S.A. Hawaii	Barbe 1964
1946	Norway	Jørstat 1946
1952	Tanzania	Wallace 1952
	Australia	Walker 1954
1953	New Zealand	Close 1958

Californian Antirrhinums (Blasdale, 1903). The rust remained unknown outside California until 1909, but from that time forward the pathogen spread rapidly round the world (Table 1). This spread has been restricted to cultivated snapdragons. Each time the rust was carried over a natural barrier, such as the Sierra Cascale range in California or the Atlantic Ocean, it spread rapidly until it reached a new barrier (Barbe, 1964).

The symptoms of disease caused by P. antirrhini were described by Doran (1921) and Green (1934). Both of these workers stated that the disease may occur on all aerial parts of plants of all ages but Close (1958) stated that the petals were not infected. The first sign of attack is the appearance of light coloured patches, which rapidly browned as uredosori form and rupture the epidermis (Green, 1934). It is the girdling of the stem by uredosori which causes the branches of the plant to wilt and die although, according to Doran (1921), it is not especially common for P. antirrhini to cause the death of the host plant. However, Blasdale (1903) observed that every season infected plants were killed by rust shortly after reaching the flowering stage. These two sets of observations may reflect a difference in regional climates, as under semi-arid conditions, injury to snapdragon plants infected by the rust was always entirely due to the drying out of rust-invaded tissues (Dimock & Baker, 1951; Walker, 1954). Nevertheless, it should be noted that in wetter regions the host tissue was damaged frequently by facultative pathogens which entered the host tissues through rust pustules (Dimock & Baker, 1951; Close, 1958). Later in the season another type of spore the teleutospore appeared. This was thicker walled and dark brown in colour. This type of spore was usually formed by rust

fungi in order to overwinter (Green, 1934).

Other effects of climate on P. antirrhini have been observed. Andres (1935) described an outbreak of the rust disease in Germany and observed the development of teleutosori at temperatures below 18.5°C followed by infection of the host plant during February and March. He also suggested that his disease records might be an under-estimate owing to the death of host plants caused by frost action. On the other hand, Moore (1940) attributed the sudden spread and destructiveness of antirrhinum rust in the U.K. to a succession of hot summers which began in 1933 and Close (1958) stated that antirrhinum rust was a warm weather disease. The optimum temperature for the life cycle of this rust was stated to be 25-29°C (Dimock & Baker, 1951). Hawker (1957) associated unusually warm summers with the production of teleutospores, a phenomenon also associated with the overwintering of P. antirrhini (Green, 1934).

The germination and infectivity of both spore types of P. antirrhini have been examined by many researchers. The optimum temperature for uredospore germination in conditions of high humidity was found to be 10°C (Doran, 1921; Mains, 1935; Chittenden, 1934) or 15°C (Yap, 1969). Peltier (1919) failed to germinate teleutospores. Doran (1921) also made numerous attempts to germinate these spores under many varied conditions, but was always unsuccessful and so concluded that the teleutospores were non functional. However, germination of teleutospores was observed for spores which had been subjected to winter conditions (Hockey, 1921; Mains, 1924; Chittenden, 1934; Green, 1941). The different results obtained in the above studies may, in part, be due to the fact that two different morphological

types of teleutospore have been found which may be physiologically different (Green, 1941; Doidge, 1941; Walker, 1954). The study of longevity of both spore types has demonstrated that teleutospores germinate most abundantly soon after maturity (Mains, 1924; Chittenden, 1934), and that uredospores can germinate 42-49 days after formation (Doran, 1921). Doran concluded that temperature had less effect than humidity on longevity as spores soon died in dry conditions. However, in a later study Walker (1924) found that uredospores remained viable for 116 days under conditions of low temperature and humidity, but as the temperature and humidity rose the longevity of the spores decreased.

The importance of temperature and humidity was evident also in infection experiments. No infection was obtained using teleutospores (Hockey, 1921; Mains 1924), so not surprisingly other workers have concentrated on the study of uredospores. Doran (1921) showed that a temperature of 10°C during the early stages of infection gave a high number of uredosori even if the plants were transferred to a higher temperature after 12 hours. This was confirmed later by Dimock and Baker (1951) who also showed that incubation at above 26°C led to the abortion of a high proportion of 24 or 36 hours-old infection, established previously at lower temperatures. Both of these studies used a low temperature for spore germination and the initial stages of infection, but subsequently raised the temperature. The optimum temperature for the growth of the fungus was found to be 21-24°C, (Dimock & Baker, 1951; Close, 1958). A completely different line of investigation into the infection process demonstrated the importance of the leaf epidermis in infection. Spore germination without tissue penetration occurred on detached leaves from which the epidermis had

been removed, but with whole leaves spore germination and tissue penetration were observed within ten days (Rossetti & Morel, 1958). This incubation period of within 8-14 days is quite usual (Peltier, 1919; Close, 1958), and so underlined that the spore development in the *in vitro* study mentioned above related to the conditions for whole plants as far as the incubation period is concerned.

As shown above, *P. antirrhini* spread rapidly around the world to infect cultivated snapdragon plants in many countries. With such a rapid spread it was natural to expect speculation concerning the means by which it was dispersed. The entry of the rust into Italy (Preti, 1935), Sweden (Palm, 1937) and South Africa (Bottomley, 1940) was attributed to seed transmission. After various investigations other workers have rejected the concept of seed transmission (Tilford, 1932; Hasselbrauk, 1937; Green, 1941). These workers did not seem to consider the possibility of viable spores carried on seed being dusted onto established plants in a nursery area. However, Baker (in Walker, 1954) considered this a distinct possibility. Walker (1954) considered the possible mode of entry of *P. antirrhini* into Australia. He found both uredospores and teleutospores on seed samples, but failed to obtain infection of susceptible seedlings using these spores. He considered that if seed transmission had been effective, the disease would have been introduced at an earlier date considering that an average of 1-2 x 10^6 uredospores were present per ounce of seed. Other possible methods by which the disease may have been spread were on infected cuttings and seedlings by wind. Walker (1954, 1955) presented strong evidence that wind borne uredospores were responsible for the initial spread of snapdragon rust in New South Wales. *P. antirrhini* was considered to have reached New Zealand by means of

wind borne uredospores carried the 1200 miles from Australia by north west winds (Close, 1958). The hypothesis of disease spread by wind is further reinforced by the discovery of two races of P. antirrhini (Yarwood, 1937). The race of rust found in New Zealand is the same as that found in Australia. Also uredospores of snapdragon rust have been shown to be liberated readily by winds in excess of 2m sec^{-1} and rainfall enhances this release (Carter et al., 1970). Although there is evidence for wind dissemination, Walker (1954) considered that the distances involved in some of the earlier outbreaks, such as that in Europe or Australia, were considerable and this may make the seed transmission hypothesis appear more feasible.

Many control measures have been tried to arrest or prevent infection of snapdragons by P. antirrhini. Changes in the general methods of cultivation, including the avoidance of high humidities and low night temperatures have been suggested (Doran, 1921; Tilford, 1932). In California early planting of snapdragons has been used so that the plants reach maturity before the rust becomes active in spring (Emsweller & Jones, 1934). Much research has been done on the use of fungicides both to arrest and to prevent disease development. Doran (1921) advocated sulphur sprays at a minimum temperature of 26°C. Bordeaux mixture was found to be ineffective under unfavourable conditions (Doran, 1921; Dimock & Baker, 1951), whereas Green (1936) found both Bordeaux and Burgundy mixtures superior to sulphur under the relatively cool moist climatic conditions prevailing in the U.K. None of these workers found these fungicides to be as effective as dithiocarbamates and sulphur fungicides which were, however, observed at temperatures rather higher than the optimum for uredospore germination found by other researchers (Jacks & Webb, 1956).

Another line of investigation has been the breeding of rust resistant cultivars. Doran (1921) found that none of the cultivars grown at that time were rust resistant, but Green (1937) obtained some cultivars resistant to the strain of P. antirrhini found in the U.K. This resistance was found to be controlled by a single dominant factor. Unfortunately, the rust resistant plants produced poor flowers and so these cultivars were used for breeding further cultivars with better flowers which could only be done by crossing with good flowering but susceptible cultivars. By 1941 Green (1941) had obtained five cultivars with good habit which were rust resistant but in America rust resistant stocks of plants had been destroyed by a new virulent race of P. antirrhini (Yarwood, 1937). This virulent race 2 rust was the one which spread to Australia and New Zealand (Walker, 1954) and all American or English cultivars of snapdragon were suceptible to this virulent form of the disease (Close, 1958).

It appeared that only race 1 of P. antirrhini was present in the U.K. and so the resistant lines developed by Green (1941) could be cultivated, but in 1969 trials on 76 of these rust resistant antirrhinums were carried out at Wisley and not one was found to be free of rust, indeed some were severely affected. Cultivars which proved to be rust resistant in this country prior to 1969 had always been susceptible to the disease when grown in the U.S.A., Australia or New Zealand due to the presence of virulent strains of the rust. As all the stocks used in the 1969 trials were affected by P. antirrhini it would seem as though either, a new virulent strain of the rust fungus had come into the country from abroad or that one or more strains of the rust already present in the U.K. had mutated to become more virulent (Brooks, personal communication). So although some

cultivars may be resistant to certain of the strains of P. antirrhini already present, if that resistance breaks down completely, then the disease could once again be as troublesome as it was in the 1930's and 1940's.

METHODS

Many different methods have been used to assess populations of leaf surface micro-organisms, but no one method has proved wholly adequate.

Direct methods of assessment using light or electron microscopy are generally too laborious for quantitative studies and are used mainly for diagnostic purposes, except when separating groups having major morphological differences. They are also helpful in determining the spatial distribution of microbes on leaves and also for studies on the habit of fungal propagules.

Because of the deficiencies of direct observation, most studies of phylloplane microbes have used techniques in which organisms are detached from leaf surfaces and cultured on nutrient media. These cultural methods present difficulties, not the least of which is associated with sampling procedures. Saprophytes are not uniformly distributed on leaf surfaces, many microbes occurring in the depressions overlying epidermal cell walls (di Menna, 1959; Ruinen, 1961). Thus it is important to define the exact area of the leaf which has been sampled. Several cultural methods have been used in phylloplane studies. Direct plating of leaf fragments was observed to be selective for fast growing organisms (Kerling, 1964; Bier, 1965). Leaf imprint on a nutrient medium would provide some measure of the distributions of micro-organisms on leaves (Potter, 1910) although they would be selective for organisms which could be easily removed from the leaf. Further the colonies developing on agar may be subject

to inter-colony competition and possibly antagonism. These factors could be minimised using the balloon print method (Rusch & Leben, 1968), although this method is less convenient to use.

Washing techniques have been used for removing microbes from leaves, the suspended microbes being plated subsequently onto nutrient media for enumeration and identification. These methods make use of the abrasive action of turbulent water for detaching propagules from leaves. The efficiency of the washing process has been shown to depend on many factors including duration of washing, temperature (Crosse, 1959), the ratio of water volume to container volume, leaf area washed and the size of individual leaf pieces (Stott, 1971). Surfactants have been used in some studies to aid the efficiency of the washing process, but their use should be carefully examined as Steiner and Watson (1965) have shown that some surfactants are fungitoxic. A decrease in fungal numbers and an increase in bacterial numbers has been observed in the presence of Tween 80 (Polyoxyethylene Sorbitan Monooleate) (Stott, 1971). Thus the main problem involved in using these agents is to distinguish between the improvement in surface sampling and the influence that the agent may exert on the subsequent development of micro-organisms in culture. It should also be borne in mind that washing methods sample propagules, e.g. bacteria, yeasts and spores of filamentous fungi, which are detached relatively easily, whereas hyphae remain largely ignored, a serious defect. Nevertheless, it seems that washing methods, notwithstanding these deficiencies, provide indications of population trends (Dickinson, 1967; Beech & Davenport, 1971).

Intact leaf pieces are usually used in washing procedures, but sometimes suspensions have been prepared from leaf macerates providing a suspension of micro-organisms which are either detached from the leaf or, if they remain still attached, are present in low numbers on the remains of individual leaf fragments. This method may possibly be subject to a lower number of variables than the washing methods. Although Hislop and Cox (1969) found no consistent differences between washing and maceration techniques, McBride (1970) obtained slightly higher counts of organisms isolated from larch leaves by maceration than he obtained by a washing technique. However, there are possible drawbacks to this technique as with other isolation methods, because Wood (1967) when reviewing a range of substances produced naturally by angiosperms highlighted the presence of some, including phenols, alkaloids, and glycosides which were active against different pathogens. As these compounds are unlikely to be inactivated during the isolation of saprophytes, care should be taken when assessing the results obtained.

In addition to the general methods, others more specialised have been used in phylloplane studies, e.g. the sporefall technique (Last, 1955a) which relies on the active discharge of spores by members of the Sporobolomycetales.

All of these methods can be criticised on some grounds and so Dickinson (1967) advocated the simultaneous use of at least two methods.

PRELIMINARY EXPERIMENTS

To ascertain which methods might prove most useful in the study of the phylloplane microflora of A. majus it was necessary to do some preliminary experiments. Leaf impressions were tried but because of their high density it was impossible to count the numbers of microbes with any degree of accuracy. Nevertheless, both this method and the sporefall technique showed that considerable differences occurred in the distribution of organisms over the leaf surface, so it seemed desirable to take whole leaf samples to ensure that the effects of the non-random distribution were not overlooked.

Series of tests were made comparing leaf washing and maceration techniques to assess some of the errors incurred.

During September 1971, leaves were removed from similar positions on several plants which had been grown in an open cold frame. Leaf samples having a surface area of about 100 cm^2 were washed for 60 minutes at room temperature in a series of different volumes of sterile water. The resulting suspensions were diluted and plated out onto nutrient media. As the use of volumes in excess of 50 ml conferred no advantage (Table 2) this volume of washing fluid contained in 100 ml flasks was used as standard in subsequent tests. When the effects of:- (1) the duration of washing and (2) the use of 0.01% Tween 80 solution were tested, it was found that the numbers of microbes washed from the leaves was increased in the presence of Tween 80, and that no significant change in numbers occurred when the washing time was increased beyond 60 minutes (Fig. 1). It was also

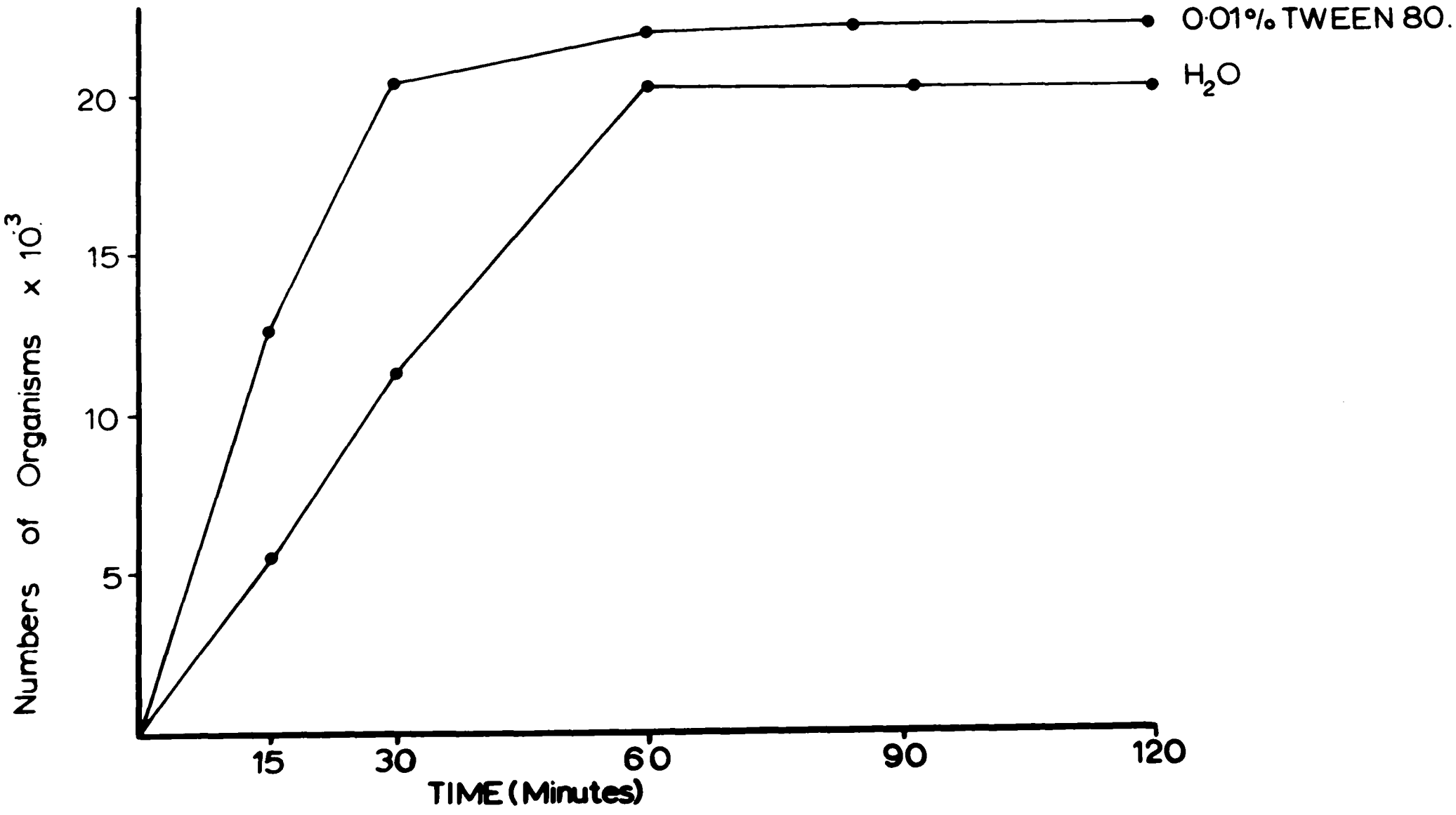

Fig. 1 Effect of duration of leaf washing in water or 0.01% Tween 80 solution on numbers of microbes isolated from leaves of A. Nanum

Table 2 Effects on numbers of microbes isolated when standard areas of leaf were washed with different volumes of water

Water volume	Total numbers of organisms isolated x 10^{3} cm^{-2}
25	15.6
50	21.2
100	22.0
200	20.2

Table 3 Comparison of numbers of microbes isolated when leaves were either washed or macerated in solutions with or without 0.01% Tween 80

	Numbers of microbes x 10^{3} isolated cm^{-2} leaf surfaces	
Fluid used	0.01% Tween 80	Water
Technique		
Maceration	23	21
Washing	24	19.5

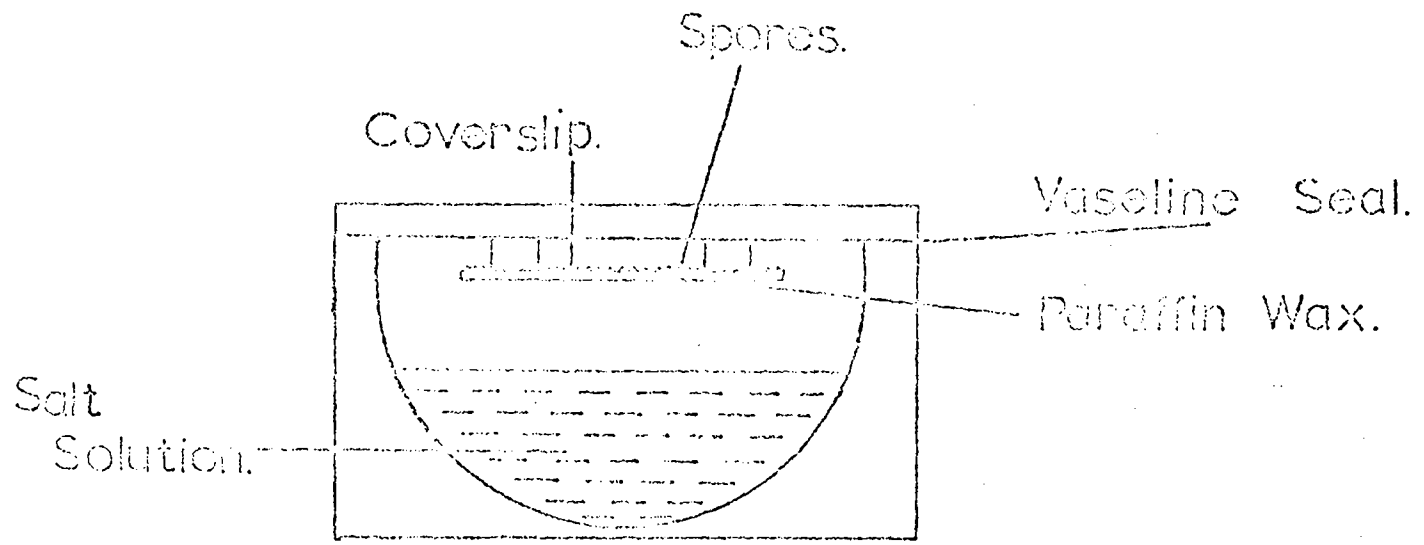

Fig. 2 Apparatus for investigating the effects of humidity on fungal spore germination

found that the most yeast and bacteria were washed from leaves within 30 minutes in the presence of Tween 80 but the numbers of filamentous fungi increased considerably until the 60 minutes washing period had elapsed.

A comparison of leaf washing and maceration was done using samples of leaves having a surface area of 100 cm^2. The leaves were either macerated in 10 ml of water or Tween 80 solution for 1 minute using a Jonke and Kunkel Ultra-turax homogeniser, or washed for 60 minutes in 50 ml of water or Tween 80 solution. Maceration did not give higher counts than the washing procedure when 0.01% Tween 80 was used as washing fluid and thus it was reasonable to adopt the latter procedure (Table 3).

Tests were done using several different isolation media and those giving the highest counts were selected for the isolation of:-

(a) filamentous fungi and yeasts: MEC containing Malt Extract agar (Oxoid) plus 50 $\mu g\ ml^{-1}$ Chloramphenicol to inhibit bacterial growth.

(b) bacteria: PYN containing Peptone Yeast agar plus 50 $\mu g\ ml^{-1}$ of Nystatin (Goodfellow et al., 1968).

One difficulty which became apparent was that antirrhinum seeds germinated poorly in the cool spring conditions occurring in Scotland. Thus it was proposed to adopt a standard nursery practice of germinating the seeds in glasshouse conditions and then transplanting the seedlings into pots before moving them outside. One problem associated with this procedure was the low number of microbes isolated from glasshouse grown plants. When leaves of such plants were sampled in the late autumn of the previous year practically no organisms were isolated using the standard washing technique

mentioned above. However, a reduction in the volume of washing fluid to 25 ml allowed meaningful plate counts of the micro-organisms isolated to be obtained, even though the efficiency of washing would be expected to be lower.

ISOLATION AND ENUMERATION OF MICRO-ORGANISMS

(1) PROCEDURE

Two cultivars of Antirrhinum majus were selected for use in this study. A. majus Nanum (Thompson & Morgan, Ipswich), a cultivar susceptible to attack by Puccinia antirrhini, (hereafter referred to as A. Nanum) and A. majus Orange Scarlet Fi hybrid tall forcing strain (Suttons Seeds, Reading), a rust resistant cultivar (hereafter referred to as A. Fi hybrid) were used. The latter cultivar has not been officially described so a short description is given in Appendix 2.

Seeds of both cultivars were sown in Levington compost and kept in a glasshouse at 15-18°C. When seedlings had grown to a suitable size for handling, they were 'pricked out' and planted in pots (100 mm square) containing University of California Mixture D2 compost (UC mix D2) (Matkin & Chandler, 1957), and allowed to establish before being transferred to a cold frame at the end of May, at which time three pairs of true leaves were visible. Samples of about 100 cm^2 were removed from leaves at the third formed node at the time of transfer to the cold frame and subsequently at two weekly intervals. Three replicate samples were obtained from each cultivar of antirrhinum, each sample being placed in a 100 ml conical flask and 50 ml of sterile 0.01% Tween 80 solution added (25 ml aliquots were used on the first

sampling date for each cultivar). These flacks were shaken for one hour at room temperature before samples of the resulting suspension were removed and diluted serially, aliquots (0.1 ml) being surface plated onto each of three replicate plates of MEC and PYN at each dilution. All plates were incubated for five days at 18°C before counting. Only plates with less than 30 colonies of filamentous fungi (Montegut, 1960), and 30-300 colonies of bacteria or yeasts (Jensen, 1968) being used for the calculation of quantitative estimates of the microbial population. Total leaf areas of each sample were assessed automatically with equipment supplied by Hayashi Denko Manufacturing Company.

In addition to the washing procedures, isolates from three upper and lower leaf surfaces were obtained from each cultivar of antirrhinum using the sporefall technique employing MEC agar.

(2) IDENTIFICATION OF PHYLLOPLANE MICRO-ORGANISMS

This analysis of the microbial population was done using a proportional subsampling procedure. Each plate which had been counted was placed on a perspex plate upon which a square grid pattern had been engraved. The organisms were isolated from squares selected using random number tables. Five filamentous fungi and five yeasts were isolated from each plate of MEC agar, and five bacteria per plate of PYN agar. A total of 45 organisms of each of the above groups was obtained for each cultivar of antirrhinum since three replicate plates from the three samples of leaves were used. Filamentous fungi were identified in two stages: in the first instance using general keys, (Zycha, 1935; Barnet, 1960; Barron, 1968; Ellis, 1971) and secondly by comparison with species monographs. The yeast isolates were

identified using the scheme of Beech *et al.* (1968) confirmed by cross reference to Lodder (1971). Bacteria were initially sorted by gram reactions. Gram positive organisms were identified using the schemes of Bergey (Breed *et al.*, 1958) and Skerman (1967). Gram negative bacteria were identified to genus using the scheme of Park and Holding (1966) and confirmed by cross reference to Bergey (Breed *et al.*, 1958).

RESULTS

(1) CLIMATIC DATA

These data were obtained from records of the Blackford Hill Observatory situated about 600 m from the sampling site at King's Buildings, Edinburgh University.

(a) Temperature

The temperature maxima and minima steadily increased from April to July, then fell slightly during August to drop more conspicuously during September (Fig. 3).

(b) Rainfall

The total monthly rainfall decreased from April to September (Fig. 3). Some rain had fallen in the 24 hour period immediately prior to sampling the antirrhinum leaves, except for samples taken on 1 and 13 June.

(c) Humidity

The meteorological observatory at Blackford Hill records relative humidity at hourly intervals but monthly summaries and mean values are not calculated routinely. The hourly record was used and processed by setting an arbitrary value of high humidity of 90% R.H. and totalling the number of hours per month for which the recorded humidity was equal to, or higher than this value (Fig. 3). This record showed that high humidities prevailed for 14% of the time prior to July, whereas during July the humidity was high for about 30% of the time. Subsequently the periods of high humidity fell to include only about 18% of the time.

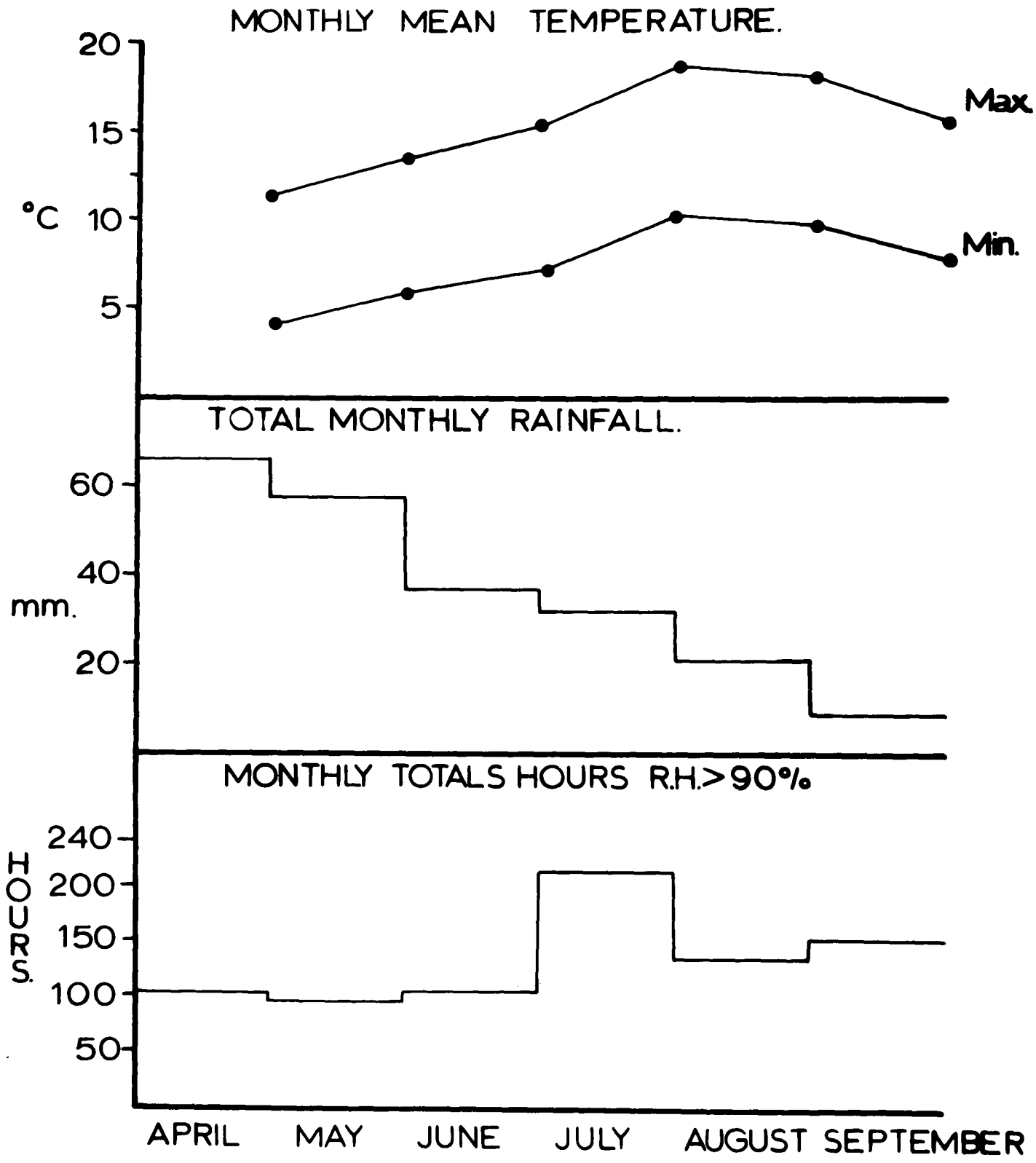

Fig. 3 Meteorological data April-September 1972:
Blackford Hill Observatory

(2) ENUMERATION OF PHYLLOPLANE MICRO-ORGANISMS FROM MAY TO SEPTEMBER 1972

Antirrhinum plants were transferred from the glasshouse to an open cold frame on 18 May for A. Fi hybrid and 29 June for A. Nanum. Plants of the latter cultivar had been planted out in May, but had been sprayed accidently with a fungicide and replacement plants were not ready for transferring to the cold frame until the end of June. Difficulties with the supply and germination of seeds of A. Fi hybrid made it impractical not to continue using the plants transferred to the cold frame in May, as these had not received chemical treatment.

Towards the end of the sampling period, 7 and 21 September for A. Fi hybrid; 21 September for A. Nanum, leaves at the positions being sampled were showing visible signs of senesence, mainly discolouration probably due to the loss of chlorophyll. The total loss of these leaves coincided with several nights of severe frosts at the end of September. P. antirrhini successfully infected A. Nanum but not A. Fi hybrid plants. By 7 September uredosori had broken through the lower epidermis of the leaves. Between 7 and 21 September the infected leaves had turned yellow and so many had fallen off that insufficient material remained for sampling and so no results were recorded for these leaves at that time.

(a) A. Fi hybrid: Numbers of micro-organisms isolated from leaves

i) Leaf washings

During the period of sampling (Fig. 4), numbers of filamentous fungi ranged from 27.4 cm^{-2} in May to 7270 cm^{-2} in September, numbers of yeasts from 6.9 to 21800 cm^{-2} and numbers of bacteria from 28.4 to 5200 cm^{-2}.

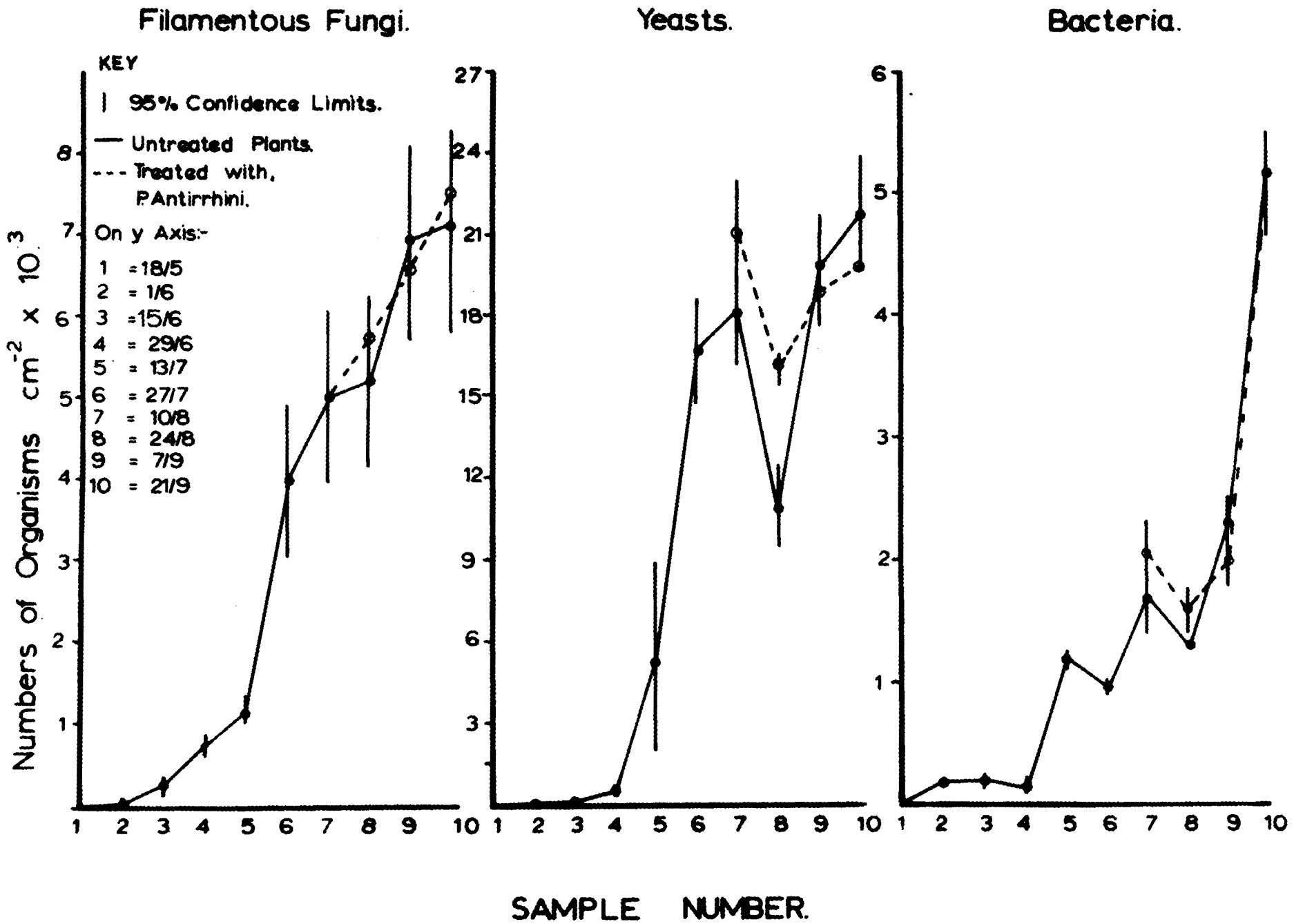

Fig. 4 Seasonal changes in numbers of filamentous fungi, yeasts and bacteria on A. Fi hybrid leaves in 1972

Filamentous fungi: Numbers of propagules isolated increased slowly during June but a significant ($p = .05$) rise from 1150 to 3420 cm^{-2} occurred between 13 and 27 July. During August the rate of increase slowed, but a further rise to 7100 cm^{-2} was observed during September. Inoculation with *P. antirrhini* did not affect numbers significantly.

Yeasts: Few yeasts were isolated until July when the numbers increased from 486 cm^{-2} on 29 June to 18200 cm^{-2} on 10 August. This rise was followed by a sharp decline but numbers recovered to give 21800 cm^{-2} by the end of September. More yeasts were usually isolated from plants inoculated with *P. antirrhini* than from uninoculated leaves, but the difference was only significant on 24 August.

Bacteria: Fewer bacteria were isolated than yeasts or filamentous fungi. After a lag phase numbers increased to 1250 cm^{-2} on 13 July and then fluctuated before increasing to 5200 cm^{-2} at the end of September. Bacterial numbers were not affected significantly as a result of inoculating leaves with *P. antirrhini*.

ii) Sporefall

This technique selectively favours the isolation of *S. roseus* although a very few colonies of white yeasts and *Cladosporium* spp. were also isolated. *S. roseus* was first isolated from the upper surface of leaves on 15 June and from the lower surface on 13 July. The difference between the two sides of leaves was also observed in the higher numbers of *S. roseus* isolated from the upper surface and the marked increase in numbers from 10 cm^{-2} on 27 July to 50 cm^{-2} on 10 August, which was not observed in isolations from the lower side of leaves.

Seasonal increases were detected earlier by the leaf washing technique than when using sporefall.

More colonies were isolated from leaves inoculated with P. antirrhini in the first sample after inoculation but differences in later samples were only slight (Fig. 5a).

(b) A. Nanum: Numbers of micro-organisms isolated from leaves.

i) Leaf washings

During the period of sampling numbers of micro-organisms showed an overall increase. Filamentous fungi increased from 38.5 cm^{-2} in June to 5100 cm^{-2} in September, numbers of yeasts from 10.5 to 19900 cm^{-2}, and bacteria from 32.8 to 600 cm^{-2} on healthy leaves (Fig. 6).

Filamentous fungi: After a short lag numbers increased slightly throughout the season to give 5100 cm^{-2} in September. P. antirrhini infection did not affect numbers isolated.

Yeasts: Numbers increased steadily from July to September. Significantly higher ($p = .05$) numbers of yeasts were isolated from leaves infected by P. antirrhini particularly after the formation of uredosori.

Bacteria: Numbers of bacteria showed an initial increase to give a peak value of 743 cm^{-2} on 10 August, followed by a sudden decrease to 360 cm^{-2} and then a slight increase during September to give a significant ($p = .05$) rise during the season. Significantly larger numbers of bacteria were isolated from leaves infected by P. antirrhini compared to the uninfected leaves.

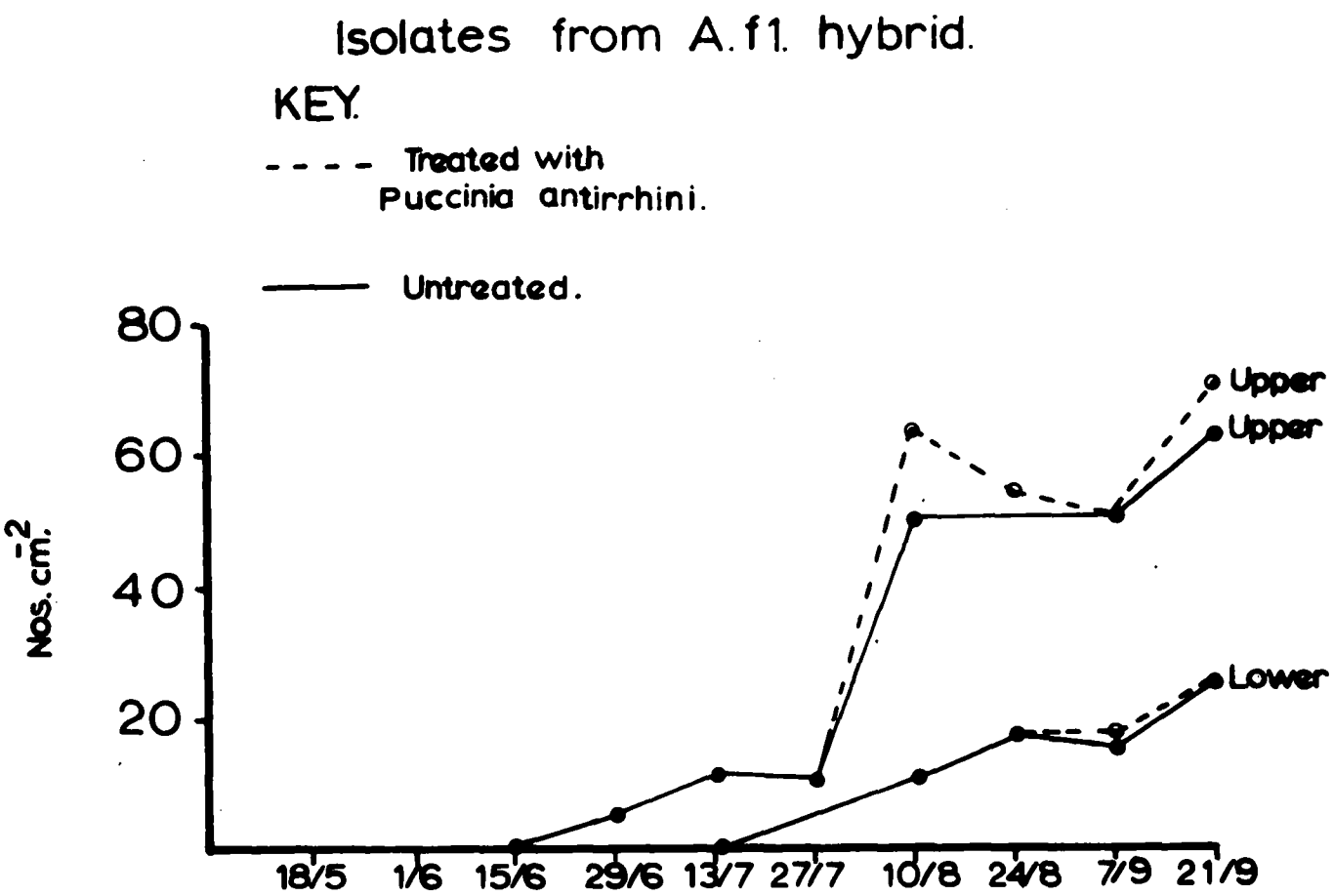

Fig. 5 Seasonal changes in numbers of S. roseus isolated by sporefall technique from a) A. Fi hybrid b) A. Nanum

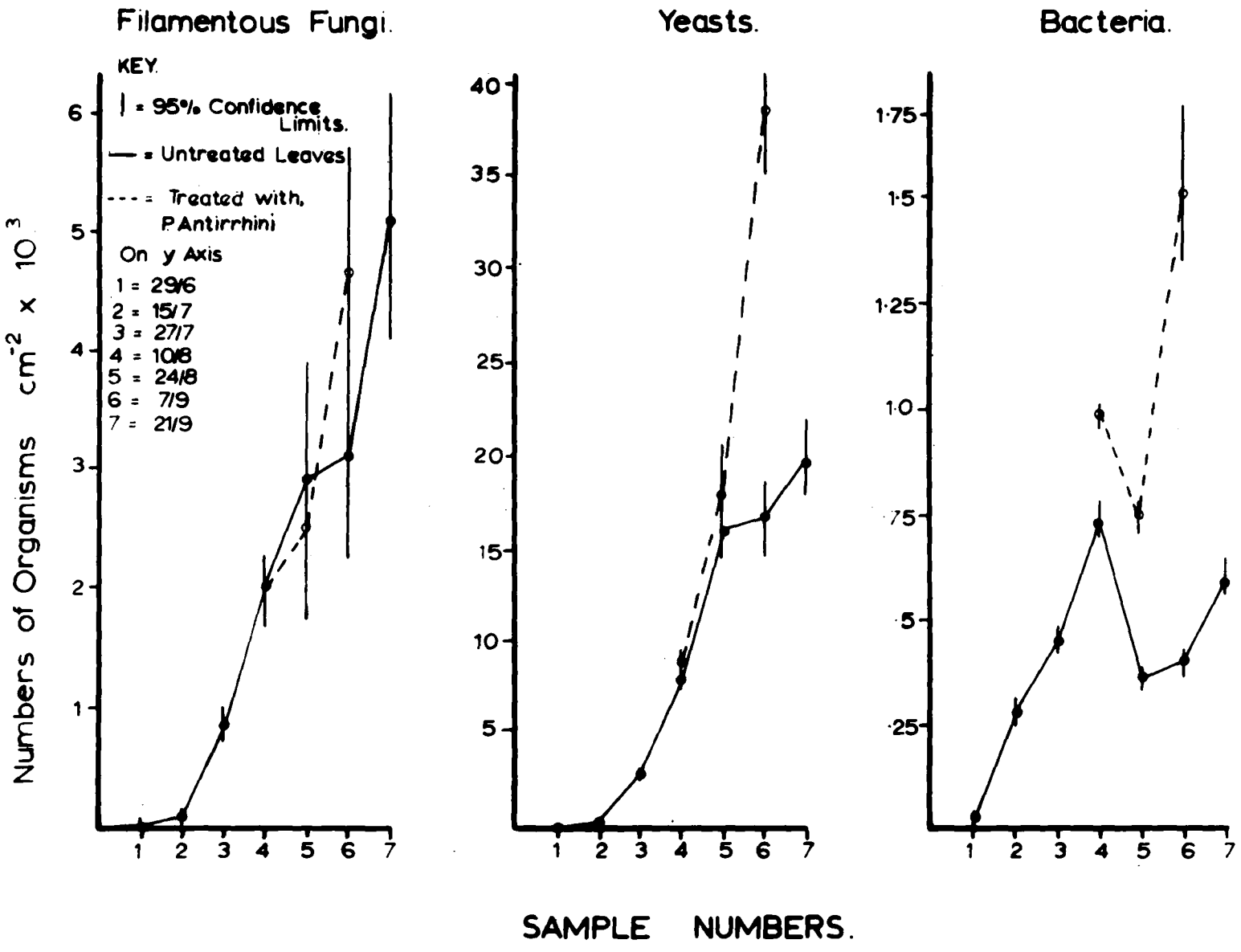

Fig. 6 Seasonal changes in numbers of filamentous fungi, yeasts and bacteria on A. Nanum leaves in 1972

ii) Sporefall

The results gained by this method (Fig. 5b) reinforce the picture shown by the results of leaf washing, in that the initial increase in numbers of yeasts on infected leaves is due to an increase on the inoculated adaxial surface whereas the major increase occurs later on the abaxial surface at the time that uredosori rupture the lower epidermis of the leaves. The pattern of colonisation on uninfected leaves is similar to that described above for leaves of A. Fi hybrid shown by this isolation technique.

(3) IDENTIFICATION OF PHYLLOPLANE MICRO-ORGANISMS

Similar microbes were isolated from both cultivars of antirrhinum (Tables 4-9).

Filamentous fungi: The most abundant form was Cladosporium cladosporioides (Fresen.) de Vries (Tables 4, 5). Cladosporium herbarum (Pers.) Link ex S.F. Gray was also present on some occasions. Numbers of Aureobasidium pullulans increased steadily throughout the season as did numbers of a white sterile mycelial colony. Epicoccum nigrum Link ex Schlect. was isolated late in the season and Botrytis cinerea Pers. ex Pers. was isolated from leaves showing visible signs of senescence. The analysis of the populations from plants inoculated with P. antirrhini showed the same overall picture on A. Fi hybrid, but on A. Nanum numbers of B. cinerea isolated on 7 September were considerably higher than those isolated from the uninoculated plants. However, rust infection accelerated the senescence of A. Nanum leaves which were shed earlier in September.

Table 4 Changing numbers of different filamentous fungi isolated from leaves of A. Fi hybrid in the period May to September 72

(Relative numbers cm^{-2} calculated from proportional sub-sampling data)

Species isolated	Sampling date									
	18/5	1/6	15/6	29/6	13/7	27/7	10/8	24/8	7/9	21/9
Aureobasidium pullulans (de Bary) Arnaud				20.0	59.0	120	180 (345)	240 (370)	350 (380)	730 (600)
Botrytis cinerea Pers. ex Pers.	2.1				43.0				45.0 (270)	58.0 (390)
Chaetomium globosum Kunze ex Fries.	1.1		38.0							
Cladosporium cladosporioides (Fresen.) de Vries	22.2	3.6	187	628	963	3800	4500 (4290)	4817 (4960)	5940 (5540)	6042 (6098)
Cladosporium herbarum (Pers.) Link ex S.F. Gray			17.0	31.0	58.0		240 (265)	60.0 (125)	98.0	
Epicoccum nigrum Link ex Schlect.								33.0 (65.0)	263 (270)	284 (302)
Fusarium spp.		0.9								
Penicillium spp.	2.1			45.0						
Sterile white mycelium			10.0	22.0	35.0		80.0 (120)	50.0 (180)	180 (140)	69.0 (110)

Figures in parenthesis refer to isolates from leaves inoculated with P. antirrhini

Table 5 Changing numbers of different filamentous fungi isolated from leaves of A. Nanum in the period June to September 72

(Relative numbers cm^{-2} calculated from proportional sub-sampling data)

Species isolated	Sampling date						
	29/6	13/7	27/7	10/8	24/8	7/9	21/9
Alternaria tenuis Nees	2.0						
Aureobasidium pullulans (de Bary) Arnaud			15.5	154 (170)	410 (460)	520 (840)	1020
Botrytis cinerea Pers. ex Pers.	9.5				(80.0)	(385)	50.0
Cladosporium cladosporioides (Fresen.) de Vries	20.0	81.0	681	1820 (1740)	2310 1830)	2460 (3300)	3900
Cladosporium herbarum (Pers.) Link ex S.F. Gray		16.0	155		65.0		
Epicoccum nigrum Link ex Schlect.					25.0 (50)	30.0 (85)	58.0
Fusarium spp.		10.0					
Mucor genevensis Lend.					50.0	70.0 (10.0)	
Penicillium spp.	7.2						
Sterile white mycelium			30.0	83.0 (90.0)	80.0 (80.0)	85.0 (85,0)	72.0

Figures in parentheses refer to isolates from leaves inoculated with P. antirrhini

Yeasts: The most abundant yeast was S. roseus which was isolated in increasing numbers throughout the season from leaves of both cultivars of antirrhinum (Tables 6, 7). The white yeasts Torulopsis sp. and Cryptococcus sp. were isolated in relatively low numbers, with the latter species being isolated more frequently later in the season. The inoculation of leaves with P. antirrhini led to an increase in the ratio of numbers of pink to white yeasts isolated. This change was greatest among isolates from A. Nanum than those from A. Fi hybrid.

Bacteria: A yellow pigmented gram negative rod probably belonging to the group Erwinia herbicola (Geilinger) Dye 1964 was the commonest isolate and with Pseudomonas sp. and Bacillus sp. accounted for most of the bacterial population (Tables 8, 9). On A. Nanum leaves infected with P. antirrhini the relative proportions of these three species of bacteria remained essentially the same. However, on A. Fi hybrid leaves inoculated with the rust E. herbicola comprised a higher proportion of the bacterial population as compared to isolates from uninoculated plants.

Table 6 Changing numbers of different yeasts isolated from leaves of A. Fi hybrid in the period May to September 1972

(Relative numbers cm^{-2} calculated from proportional sub-sampling data)

Species isolated	Sampling date									
	18/5	1/6	15/6	29/6	13/7	27/7	10/8	24/8	7/9	21/9
Cryptococcus sp.						60.0	85.0 (100)	145 (110)	1050 (1340)	4200 (2320)
Sporobolomyces roseus Kluyver & van Niel	0.9	58.1	72.0	372	5047	15500	17000 (19750)	10140 (15380)	15900 (15500)	15100 (16100)
Torulopsis sp.	6.0	33.5	31.0	114	430	1110	1115 (1250)	675 (510)	2750 (2460)	2500 (1380)

Figures in parentheses refer to isolates from leaves inoculated with P. antirrhini

Table 7. Changing numbers of different yeasts isolated from leaves of A.Nanum in the period June to September, 1972.

(Relative numbers cm^{-2} calculated from proportional sub-sampling data).

Species isolated	Sampling date						
	29/6	13/7	27/7	10/8	24/8	7/9	21/9
Cryptopoccus sp.				120 (100)	180 (250)	650 (1900)	2100
Sporobolomyces roseus Kuyver & van Niel		200	2620	6200 (7800)	14800 (16800)	15350 (32200)	16100
Torulopsis sp.	10.5	45.0	320	1580 (1300)	1020 (1550)	900 (2900)	1700

Figures in parentheses refer to isolates from leaves inoculated with P. antirrhini.

Table 8. Changing numbers of different bacteria isolated from leaves of A. Fi hybrid in the period May to September 1972.
(Relative numbers cm^{-2} calculated from proportional sub-sampling data).

Species isolated	Sampling dates									
	18/5	1/6	15/6	29/6	13/7	27/7	10/8	24/8	7/9	21/9
Achromobacter sp.										(60.0)
Bacillus sp.	4.6	3.0	84.0	62.0	104.5	51.0	68.0 (215)	60.0 (230)	738 (470)	1352 (1360)
Erwinia herbicola (Geilinger) Dye 1964	10.4	88.0	44.0	38.0	721.5	680	1473 (1280)	1134 (950)	753.5 (1100)	2589 (2370)
Erwinia sp.	6.5	67.0	15.0		222	82.0	63.5 (125)	43.0	383.5 (60.0)	153 (240)
Micrococcus sp.	8.5									158 (170)
Pseudomonas sp.		35.5	55.0	53.0	109	157	95.5 (480)	65.0 (420)	525 (370)	948 (870)
Red pigmented Gram+ rods.						48.0				

Figures in parentheses refer to isolates from leaves inoculated with P. antirrhini.

Table 9. Changing numbers of different bacteria isolated from leaves of A. Nanum in the period June to September 1972.
(Relative numbers cm^{-2} calculated from proportional sub-sampling data).

Species isolated	Sampling dates						
	29/6	13/7	27/7	10/8	24/8	7/9	21/9
Bacillus sp.	15.4	93.0	33.0	125 (110)	90.0 (150)	114 (220)	173
Erwinia herbicola (Geilinger) Dye 1964	10.8	143	287	420 (610)	205 (430)	220 (1050)	260
Erwinia sp.	6.6			(140)	35.0 (70.0)	(55.0)	
Micrococcus sp.						10.0 (10.0)	30.0
Pseudomonas sp.		51.0	132	198 (140)	30.0 (90.0)	56.0 (195)	137

Figures in parentheses refer to isolates from leaves inoculated with P. antirrhini.

DISCUSSION

The data obtained during 1972 on the saprophytic phylloplane microflora of the two cultivars of A. majus presented a complex picture. An overall increase in the numbers of micro-organisms per unit area of leaf occurred during the growing season and associated with this were some changes in the species composition of the microflora isolated.

Although the number of micro-organisms increased on the leaves of both cultivars (Figs. 4, 6) during the growing season, the shape of the curves for any one group of organisms is slightly different for each of these cultivars. The main difference lies in the initial lag phase, observed in the increase in the numbers of organisms isolated from A. Fi hybrid, which was curtailed in the data obtained for A. Nanum. This difference could be due to plant cultivar, plant age, or the environmental conditions prevailing at the time of planting out. Certainly the phase of rapid increase in the numbers of micro-organisms isolated from A. Fi hybrid coincide with an increase in both the mean maximum and minimum temperatures (Fig. 3) and a rise in the duration of high humidity conditions (Fig. 3) from 108 to 218 hours. Such conditions have been shown to enhance the growth of bacteria (Leben & Daft, 1967) and some fungi (Pady et al., 1969). This period also coincided with the time at which fresh plants of A. Nanum were transferred to the cold frame and so the occurrence of favourable environmental conditions at this time might help to explain the difference in the initial rate of colonisation.

Apart from these differences in the initial lag phase, the increase in the numbers of organisms was similar for both cultivars of antirrhinum. The major grouping of micro-organisms appeared to arrive on the leaf at the same time unlike the true successional pattern of arrival observed on some plants (Ruinen, 1961; McBride, 1970). This might be due to the procedure of growing the seedlings in the greenhouse before planting out which might not allow an initial bacterial population to build up on the leaves, from a seed borne inoculum (Leben & Daft, 1966) in the dry conditions prevailing in the greenhouse (Leben & Daft, 1967).

During July the numbers of filamentous fungi increased rapidly. The isolations made at this time showed that C. cladosporioides accounted for the major part of this increase. July was a month of moderate temperature, low rainfall and periods of high humidity. Such conditions have been shown to be optimal for the sporulation and dispersal of conidia of Cladosporium spp. (Pady et al., 1969). Also some workers (Hirst, 1953; Harvey, 1967) have shown that during July Cladosporium spp. form a very large part of the airspora under dry conditions. It would appear, then, that the increase in numbers of C. cladosporioides observed on antirrhinum leaves was consistent with an increase in deposition from the air. Nevertheless, Pugh and Buckley (1971a) found actively growing colonies of Cladosporium spp. on the leaves of sycamore, some of which were sporulating at this time and therefore, it may well be that the increase in the numbers of C. cladosporioides isolated was attributable both to a high rate of deposition, and also to colonisation and sporulation. Support is also provided by occasional observations on antirrhinums where, in another study not described here, sporulating colonies were observed

with increasing frequency as the growing season progressed.

The other species of filamentous fungi isolated in reasonably high numbers were E. nigrum and A. pullulans. E. nigrum was isolated late in the season. This pattern has been observed previously (McBride, 1970; Pugh & Buckley, 1971a). A. pullulans was isolated about four to six weeks after the beginning of the experiment. Other workers have isolated this fungus from very young plants (Hudson & Webster, 1958) or even from unopened buds (Pugh & Buckley, 1971b; Warren, personal communication). The absence of this ubiquitous organism from isolations early in the season may be due to the initial growth of the plants under greenhouse conditions where the density of the airspora is low (Leben & Daft, 1967).

Leaf inoculation with uredospores of P. antirrhini did not result in any significant changes ($p = .05$) in numbers of fungal propagules isolated. Nevertheless on A. Nanum the rapid increase in numbers of fungi, especially of C. cladosporioides, at the end of the season could be associated either with the effects in the change in nutrient status of the leaf surface due to the rupturing of the epidermis by the uredosori or else, to the earlier leaf senescence observed. The latter has been shown to lead to an increase in the numbers of Cladosporium spp. (Kerling, 1964).

Yeast populations isolated from antirrhinums increased steadily after an initial lag, except for the isolation from A. Fi hybrid on 24 August, until the end of the season. Stout (1960) found an initial lag in the increase of the numbers of yeasts on pasture plants. Di Menna (1959) suggested that this pattern was due to the influence of temperature, while Last (1955a) correlated this pattern with the

occurrence of the half life of the leaves in cereals and nutrient available on the leaf surface. In this study whilst the temperature increased appreciably during the time of maximum increase in yeast numbers in July, it is possible that the differences in the nutrient status of the leaf surface between the two cultivars of antirrhinum (p. 107) could account for the observed difference in the length of the lag phase. However it should not be overlooked that the observed increases could have been partially due to deposition of yeast spores from the atmosphere, as large numbers of these spores have been observed in the airspora in July and August (Last, 1955b; Hamilton, 1959).

Different methods have been used to study yeasts on the phylloplane. A washing technique was used by di Menna (1959) to observe both white and pink yeasts and Last (1955a) used the sporefall method to study the seasonal incidence of S. roseus. In this study both of these methods were used. These showed that S. roseus comprised the major part of the yeast population, especially on the upper surface of leaves, but the two methods detected differences in the time at which the main increase in yeasts began (Table 10). The results obtained using the sporefall method show an increase that began about 14 days after that shown by the washing technique. One explanation for this result might be that using the washing technique, numbers of viable propagules are observed. These propagules may be single cells or aggregations of cells. On the other hand, the sporefall method may give only a measure of the number of yeast colonies rather than individual cells. Alternatively, it is possible that the ratio of the numbers of colonies, developing on agar in the sporefall method, to the actual colony number on the leaf may not remain constant. Thus

the initially low numbers of S. roseus colonies isolated may reflect the physiological state of the yeast, especially in terms of colony establishment. Last (1955a) produced several arguments against this hypothesis of a changing differential in the numbers of colonies on leaves to the numbers of colonies isolated and Buller (1933) showed that the interval between spore germination and subsequent spore production was only 24 hours under summer temperature conditions. These papers would seem to preclude the idea that there might be a long lag phase before ballistospore formation commenced. Nevertheless it is possible that the numbers of yeast cells might increase at a faster rate by simple division than by ballistospore formation, especially when an acceleration in the increase of cell numbers occurs. This might explain the higher ratios of the numbers of yeasts isolated by the different methods on 27 July (Table 10).

The white yeasts Cryptococcus sp. and Torulopsis sp. were also isolated. The numbers of white yeasts increased later in the season and a change in the relative abundance from Torulopsis sp. to Cryptococcus sp. occurred at the same time (Table 6, 7). This increase in numbers may be part of the pattern of low numbers of white yeasts isolated in summer and higher numbers in winter observed by other workers (di Menna, 1959; Hislop & Cox, 1969). Similar changes in the relative abundance of Torulopsis spp. and Cryptococcus spp. have been observed in isolations from larch leaves (McBride, 1970).

Treating the plant leaves with uredospores of P. antirrhini resulted in an increase in the population size of yeasts on the leaves of A. Nanum. Only on one occasion (24 August) was any significant ($p = .05$) difference observed in the numbers of yeasts

Table 10. S. roseus: Ratio of numbers isolated from leaves of A. Nanum and A. Fi hybrid by sporefall method to numbers isolated by leaf washing.

Cultivar	Sampling data									
	18/5	1/6	15/6	29/6	13/7	27/7	10/8	24/8	7/9	21/9
A.Fi hybrid	0:0.9	0:58.1	0:72	1:74.4	1:459	1:1033	1:283 (1:271)	1:151 (1:217)	1:245 (1:231)	1:174 (1:170)
A.Nanum	-	-	-	0:0	0:200	1:524	1:248 (1:120)	1:269 (1:233)	1:265 (1:190)	1:198

Figures in parentheses refer to isolates from leaves inoculated with P. antirrhini.

isolated from inoculated leaves of A. Fi hybrid compared to the numbers from the untreated leaves and no change in the relative abundance of species was observed. The increase in yeast numbers on A. Nanum was due mainly to the increase in the numbers of S. roseus shown by both washing and sporefall techniques. The results from the sporefall method give some precise details of the population changes in S. roseus. The uredospores of P. antirrhini were deposited on the adaxial surface of the leaves. An increase in the numbers of S. roseus on this surface was observed after 14 days. This may possibly have been due to the leaching of nutrients from the uredospores and germ tubes which has been observed previously (Shaw, 1963). The yeast population on the adaxial surface tended to remain at this higher level. On the abaxial surface of the leaves a vast increase in numbers occurs after the uredosori had ruptured, the lower epidermis presumably exposing damaged epidermal cells and increasing the amount of nutrients available. This increase in numbers is about tenfold, 9 colonies cm^{-2} were isolated from healthy leaves and 99.5 cm^{-2} from infected leaves. A similar increase in numbers of S. roseus on rust infected antirrhinums had been observed by Last (1970).

The numbers of bacteria isolated showed similar differences in the rate of increase between the cultivars of antirrhinum as was found for the previous two groups of micro-organisms. The arguments relating to the presence of an initial lag phase, observed only in the isolations from A. Fi hybrid, have been discussed above. After this period, although an overall increase in numbers was observed during the season, this increase was by no means regular. In the case of A. Nanum a peak was reached on 29 June, after which the numbers declined, but recovered slightly during September (Fig. 6). The data

for bacteria on A. Fi hybrid shows a pattern of increase with only minor fluctuations occurring to reach a peak of 5×10^3 cm^{-2} in September. These fluctuations in numbers could be due to the very marked response which bacteria exhibit towards changing environmental conditions, especially moisture, in the forms of humidity and rainfall. The low bacterial populations observed on crop plants in Ohio has been attributed to the low relative humidity which prevails in that area (Leben & Daft, 1967). Jensen (1971) observed that the bacterial population on beech leaves was smaller during dry summers. It is not only constant low humidity that affects bacteria, as it has been shown that fluctuating humidity can reduce the size of bacterial populations (Hatch & Dimock, 1966). Rain can also exert an effect by washing organisms from the leaf surface (di Menna, 1959; Ruinen, 1961). On the other hand a rapid increase in bacterial numbers can occur if leaves remain wet for 24 to 48 hours (Leben & Daft, 1967). Such changes in environmental conditions could easily have caused the fluctuation in the bacterial population observed here. Even within this variability certain trends may be seen. The numbers of bacteria rose sharply at the onset of leaf senescence. This was more pronounced in the isolation from A. Fi hybrid although a slight rise in numbers did occur on A. Nanum during September. McBride (1970) observed similar increases in bacterial numbers on senescing larch leaves.

The composition of the bacterial populations isolated from the two cultivars of antirrhinum were similar (Tables 8, 9), but these did not reflect the composition of the airspora, unlike the populations of filamentous fungi and yeasts. The dominant components of the bacterial airspora are gram positive rods, bacilli and micrococci (Fleming, 1908; Zobel, 1942; Pady & Kelly, 1953). Of these only a

Bacillus sp. was found in any numbers on antirrhinum leaves. The most abundant bacterium was a yellow pigmented gram negative rod shaped organisms. Similar pigmented bacteria have been found to predominate the bacterial populations of larch (McBride, 1970) and beech (Jensen, 1971). The nomenclature of these yellow pigmented organisms is complicated as both Xanthomonas trifolii (Huss) James and Erwinia herbicola have been shown to be synonymous. The whole question of these pigmented Erwinia-like organisms has been studied and it is proposed that many of these organisms be named as varieties of Erwinia herbicola (Dye, 1969). There may still be difficulties in that the gram reaction of these Erwinia-like organisms is somewhat variable (Billing & Baker, 1963). If a gram positive strain is isolated, the key of Bergey (Breed et al., 1958) could lead one to the family Brevibacteriacea. However, in this study over 95% of the yellow isolates were gram negative and the remainder gram variable, but otherwise indistinguishable and have thus been classed as E. herbicola.

Leaf treatment with P. antirrhini led to an increase in the bacterial population on A. Nanum but no significant (p. = .05) effect was observed on the leaves of A. Fi hybrid. The species composition and the relative abundance of each species on the treated leaves did not change from that found on the untreated leaves. The numbers of bacteria increased rapidly after uredosori had ruptured the lower epidermis of the leaf. The factor by which the numbers increased was similar to that shown by the yeast population although the numbers of organisms involved were much smaller.

The different effects observed on the leaf saprophytes following the application of P. antirrhini uredospores to antirrhinum leaves reflects the relative susceptibilities of the cultivars used. Although the numbers of bacteria and yeasts on the leaves of A. Fi hybrid may have risen slightly as judged from the values of the means, the variation inherent in observations of this nature was such that no significance could be attached to so small a change. On the other hand, the yeasts and bacteria on A. Nanum leaves did increase in number on infected leaves especially after the uredosori had ruptured the lower epidermis of the leaf.

The activities of both pathogen and saprophytes may have affected the host plant as the infected leaves senesced about 10 to 14 days earlier than healthy leaves of the same age. This accelerated senescence may have been due to the infection by P. antirrhini, as Blasdale (1903) observed that all infected antirrhinum plants died shortly after they had reached the flowering stage. Alternatively the increased activity of the higher numbers of saprophytes may have caused the earlier senescence as has been observed in barley leaves (Skidmore & Dickinson, 1973).

This survey of the saprophytic phylloplane microflora of the two cultivars of A. majus revealed that a similar range of organisms occurred on the leaves of both cultivars. Although direct comparisons of the numbers of microbes isolated in each case were not possible, owing to the reasons detailed above, by the end of the season the size of the total population of micro-organisms was of the same order of magnitude for healthy leaves. The organisms which occurred in the greatest abundance on both cultivars were C. cladosporioides and S. roseus. These organisms were selected for more detailed studies on leaf colonisation and any possible interactions with the pathogen P. antirrhini.

CHAPTER 2 SPORE PRODUCTION, GERMINATION AND GROWTH OF SOME FUNGI ISOLATED FROM THE PHYLLOPLANE OF A. MAJUS

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SPORE PRODUCTION, GERMINATION AND GROWTH OF SOME FUNGI ISOLATED FROM THE PHYLLOPLANE OF A. MAJUS

INTRODUCTION

Cladosporium cladosporioides (Fresen) de Vries and Sporobolomyces roseus Kluyver & van Niel were selected for further studies of leaf surface colonisation and microbial interactions as these microbes were the most abundant saprophytes isolated during the survey of the antirrhinum phylloplane (p. 40). Also both S. roseus (McBride, 1971; Fokkema, 1973) and Cladosporium spp. (Newhook, 1957; Bhatt & Vaughan, 1962, 1963; Diem, 1969a; Fokkema, 1973) were observed to be antagonistic to leaf pathogens on several plants.

Although P. antirrhini has been studied by other workers their data concerning the optimum conditions for uredospore germination and plant infection are somewhat contradictory (Doran, 1921; Moor, 1940). Other information dealt with the effects of temperature and humidity on C. cladosporioides and S. roseus (Last, 1955a; Stott, 1971; Diem, 1971), but in order to optimise conditions used in later experiments it was thought advisable to test the differing isolates obtained from antirrhinum leaves.

METHODS

1) GERMINATION OF P. ANTIRRHINI AND C. CLADOSPORIOIDES

The assessment of germination is difficult in that the germination process includes not only the morphological and physiological changes occurring within the confines of the spore wall but also the protrusion of the germ tube from the spore wall and the elongation of this germ tube. The difficulty lies in the problem of devising methods whereby the progress of each stage may be measured rapidly and accurately as a routine procedure. The use of internal changes as an assessment of germination have scarcely been used in routine work on fungi. Changes in respiratory activity have been observed in germinating spores (Goddard & Smith, 1938; Mandels et al., 1956), the measurement of which should be practicable in the study of a single species in isolation, but the interpretation of such measurements in mixed cultures would be extremely difficult.

Although the electron microscope has been used to determine the exact moment at which the germ tube begins to protrude from the spore wall (Hawker & Hendy, 1963), such techniques are inconvenient for routine work and most workers have preferred to employ an arbitrary criterion based on the appearance of an easily visible germ tube. One such criterion is the acceptance that a spore has germinated "if the length of the germ tube exceeds half the minor diameter of the spore" (American Phytopathological Society, 1943). Other workers have defined the spore as germinated when the germ

tube is as long as it is broad (Manners, 1949; French, 1961; Manners & Hossain, 1963). This latter represents, in many fungi, the earliest stage at which one can be certain, after rapid examination under the light microscope, that a spore has germinated. The earlier definition given above covers not only germination, but also a considerable amount of germ tube growth (Manners, 1966).

Germ tube growth is usually measured with the aid of some form of ocular micrometer. This procedure is very laborious except when germ tubes are very short. Photography or use of projection microscopy followed by measurement with a map measurer enables reasonable numbers of long or convoluted germ tubes to be measured. The stage at which a germ tube becomes vegetative mycelium is not easy to define as the transition, except in the cases of obligate pathogens, is often gradual. Criteria based on the source of nutrient for germ tube growth may be physiologically sound but are of little value in assessment work, as the extent to which nutrient is obtained from a medium depends on the nature of that medium (McCallan et al., 1954). Criteria based on the rate of hyphal extension may differ markedly between different fungi (Plomley, 1959). Also the variation in germination rate between spores means that no single set of criteria would be applicable universally and so it would appear that separate sets of criteria should be defined for each species examined (Manners, 1966).

The standardisation of the methods used in the production of spores for germination experiments are critical to the production of spores exhibiting a similar response to different treatments. The effects of the conditions of spore production on subsequent spore germination have been discussed for culturable microbes (McCallan & Wilcoxon, 1940a) and for obligate pathogens (Manners & Hossain, 1963).

Procedures used in these germination trials:-

(a) PRODUCTION OF SPORES OF P. ANTIRRHINI AND C. CLADOSPORIOIDES

Uredospores of P. antirrhini were produced on A. Nanum grown in glasshouse conditions. These hosts were inoculated and subsequently kept in slight shade at 15–18°C and with intermittent misting to maintain high humidity. Mature uredospores were collected from open sori about 10 days after they had erupted through the epidermis on the abaxial leaf surface (Walker, 1954).

Conidia of C. cladosporioides were harvested 10 days after inoculating spore suspensions onto plates of Czapeck Dox agar (Oxoid) which were incubated in darkness at 25°C.

(b) GERMINATION OF DRY SPORES:

The method described by Manners and Hossain (1963) was used to determine effects of humidity at different temperatures, the apparatus being shown in Fig. 2. P. antirrhini uredospores were collected dry by tapping infected leaves. C. cladosporioides conidia were collected dry by gently brushing the surface of a culture growing on agar. Spores were applied to sterile

coverslips via a settling tower (250 mm long) used to ensure their uniform distribution on coverslips (McCallam & Wilcoxon, 1940b). Wells of staining dishes were filled with saturated salt solutions to give the required humidity, note being taken of the influence of temperature (O'Brien, 1948). Three replicates were done for each treatment which were observed after a 48 hour period of dark incubation at the required temperature. The spores were examined in situ using a microscope equipped with a green filter (OGRI) to enable the spores to be seen clearly. Two hundred spores in each replicate were examined using the criterion that the germ tube should be as long as it was broad before a positive germination reaction was recorded (Manners, 1949).

(c) GERMINATION OF SPORES IN THIN LIQUID FILMS

Uredospores of P. antirrhini were collected by tapping leaves bearing open uredospori over open sterile wide-necked vials and then suspending in 10 ml of sterile deionised water. After agitation on a wrist action shaker, to break up clumps of spores, the density of suspensions was assessed using a haemocytometer. Subsequently spores were centrifuged at 3500 rpm for 10 minutes (temperature $5^{o}C$), the supernatant decanted off, and the spores resuspended in a volume of deionised water to give the required spore density, (500 spores per droplet of P. antirrhini and 5×10^{3} spores per droplet of C. cladosporioides unless otherwise stated). This was checked before being used.

Conidia of C. cladosporioides were collected by pipetting 10 ml of sterile deionised water onto colonies growing in petri dishes.

The colonies were stroked with a sterile glass rod and the suspension of conidia and hyphal fragments decanted into sterile vials which were shaken for 10 minutes. The resulting suspension was filtered through a sterile sintered glass filter (No. 2) to remove any fragments of mycelium. The spore suspension was shaken and samples removed to a haemocytometer slide for assessment of spore density. The spore suspension was then centrifuged and the supernatant poured off. The spores were resuspended in a volume of sterile deionised water to give the required spore density which was checked using a haemocytometer. The above washing procedure removes interfering traces of the original growth medium and also gives uniform spore suspensions (McCallan & Wilcoxon, 1940a).

Aliquots (0.05 ml) of spore suspension were transferred to clean sterile glass slides using a micro-syringe. The slides were put on bent glass rods in humidity chambers (glass petri dishes lined with wet sterile filter paper and sealed with parafilm), and droplets of spore suspension did not dry out in any of the conditions used in these tests. Three replicate slides were subjected to each particular treatment in all tests, and 200 spores were assessed for germination and the lengths of 50 germ tubes measured, using an ocular micrometer, per replicate. The statistical analysis of the results from germination experiments was done using the 'X^2' test (McCallan & Wilcoxon, 1932). Data for germ tube lengths was analysed using the non parametric Mann Whitney 'U' test (Siegel, 1956) so allowing for non normal distributions.

i) Effect of temperature

Spore suspensions were incubated for 48 hours in darkness at different temperatures.

ii) Effect of light

C. cladosporioides germination was assessed after incubation in light (6450 Lux) for 24 or 48 hours at the optimum temperature compared with the dark incubated control.

Two experiments on the effects of light were done using P. antirrhini:-

a) Incubation for 48 hours at optimum temperature under continuous light of two intensities:

1) 3220 Lux 2) 6450 Lux

b) Incubation for 24 hours at optimum temperature under either of these intensities of light followed by further incubation for 24 hours in darkness at the same temperature. Germination was assessed after each 24 hour period.

iii) Effect of spore density

Effects of spore density were assessed after incubating in darkness for 48 hours at the optimum temperature observed in (i).

iv) Effect of spore age

In this experiment uredospores of P. antirrhini were removed from the uredosori at different time periods after the sorus had erupted through the abaxial surface of the plant leaf, and their ability to germinate tested.

v) Rate of spore germination

The rate of germination was measured by assessing the germination at intervals of six hours after incubation in darkness at the optimum temperature.

vi) Effect of exogenous nutrients

Glucose, sucrose and fructose, which have been found to be major carbohydrate components of leaf leachates (Nykvist, 1963; McBride, 1970; Purnell, 1971) were tested. The procedure used was essentially similar to that already described except that spores were resuspended, after washing and centrifugation, in the test solutions containing 1 mM of one of the sugars. This concentration was similar to that found by Nykvist (1963), between the 0.25 mM found by Purnell (1971) on swede leaves and the high concentration of 5M obtained from droplets placed in seed cavities of beans (Deverall, 1967).

2) GROWTH OF S. ROSEUS

The growth of yeasts may be measured in terms of increase in cell number and cell size. However, conditions which may be optimal for increase in cell size may not be optimal for cell number increase. Merritt (1966) observed that the cell size of a distillers yeast increased most rapidly at 35°C but maximum cell numbers occurred at 30°C. As the purpose of the present experiments was only to obtain basic information on the conditions which might favour the growth of S. roseus, rather than to examine in detail the various measures of yeast growth under cultural conditions in test tubes, a simple comparative approach was adopted for experiments on any one environmental condition.

a) PRODUCTION OF YEAST CELLS FOR USE IN EXPERIMENTS

Cultures of S. roseus were grown in darkness in petri dishes of Malt Extract agar (Oxoid) at 18°C. A loopful of the yeast was

removed from a seven day old colony and transferred to 10 ml of sterile deionised water in a screw top vial. The vial was agitated on a wrist action shaker for 10 minutes to break up cell aggregations before measuring the density of the suspension with a haemocytometer. The yeast suspension was then centrifuged at 4500 rpm for 15 minutes at 4°C, the supernatant decanted off to remove any interfering traces of the original growth medium. The yeast cells were resuspended in fresh sterile deionised water to give the required concentration of cells which was checked subsequently.

b) EFFECTS OF TEMPERATURE ON THE GROWTH OF S. ROSEUS

Aliquots (0.1 ml) of yeast suspension were transferred to test tubes each containing 10 ml sterile Malt Broth (Oxoid). After thorough mixing with an orbital mixer the turbidity of the suspensions was measured using a spectrophotometer (Evans Electroselenium Limited). Fifty tubes of media were incubated in the dark at each temperature. At intervals of 12 hours three tubes were removed from each temperature regime and their turbidity measured. The increase in turbidity of the solutions was used as a measure of overall growth, which although it cannot distinguish between increase in cell size and numbers of cells does enable the effects of temperature on growth to be observed on a comparative basis. This process was continued until either no further increase in turbidity was observed or the seven day period of the experiment was completed.

c) EFFECTS ON YEAST GROWTH OF DIFFERENT CARBOHYDRATE SOURCES

Aliquots (0.1 ml) of suspension were used to inoculate tubes containing 15 ml of 1 mM solutions of glucose, sucrose or fructose and a control containing only water. The inoculum was examined using a dilution plate count in which 0.1 ml aliquots were surface inoculated onto Malt Extract agar (Oxoid) and incubated for five days at 18°C. Three plates for each dilution and replicate sample were set up. The size (length and diameter) of 30 cells from each sample was measured, using an ocular micrometer on a phase contrast microscope, so that the total volume of cells could be estimated. Using this procedure, five replicates of the yeast suspension from each carbohydrate treatment were examined after both 24 and 48 hours incubation in darkness at 18°C.

(d) EFFECT OF LIGHT ON GROWTH OF S. ROSEUS

In parallel with carbohydrate tests done in the dark, five replicate tubes of the 1 mM glucose treatment were illuminated by warm white fluorescent tubes and tungsten lights (6450 Lux) during incubation. Dilution plate counts and cell measurements were made after both 24 and 48 hours incubation.

RESULTS

(1) GERMINATION OF P. ANTIRRHINI

(a) EFFECTS OF HUMIDITY

Germination only occurred at humidities in excess of 95% R.H. (Fig. 7). A higher percentage germination was recorded at 10°C compared to the other temperatures used.

(b) TEMPERATURE

The optimum temperature for germination was 10°C (Fig. 8), and variation of 2°C either side of this temperature lead to a reduction in germination of about 45%.

(c) LIGHT

Light significantly inhibited ($p = .05$) both germination and germ tube growth. The effect was greater in conditions of higher light intensity (6450 Lux) as compared to light of 3220 Lux. The reversible nature of this effect was observed in the experiment in which uredospores were incubated in light conditions for 24 hours followed by further incubation in the dark for 24 hours. After this period both germination and germ tube length had increased to levels which were not significantly different ($p = .05$) from those observed in the 24 hour dark control. (Fig. 9)

(d) SPORE DENSITY

When concentrations of uredospores reached 10^4 cm^{-2} germination was appreciably reduced (Table 11).

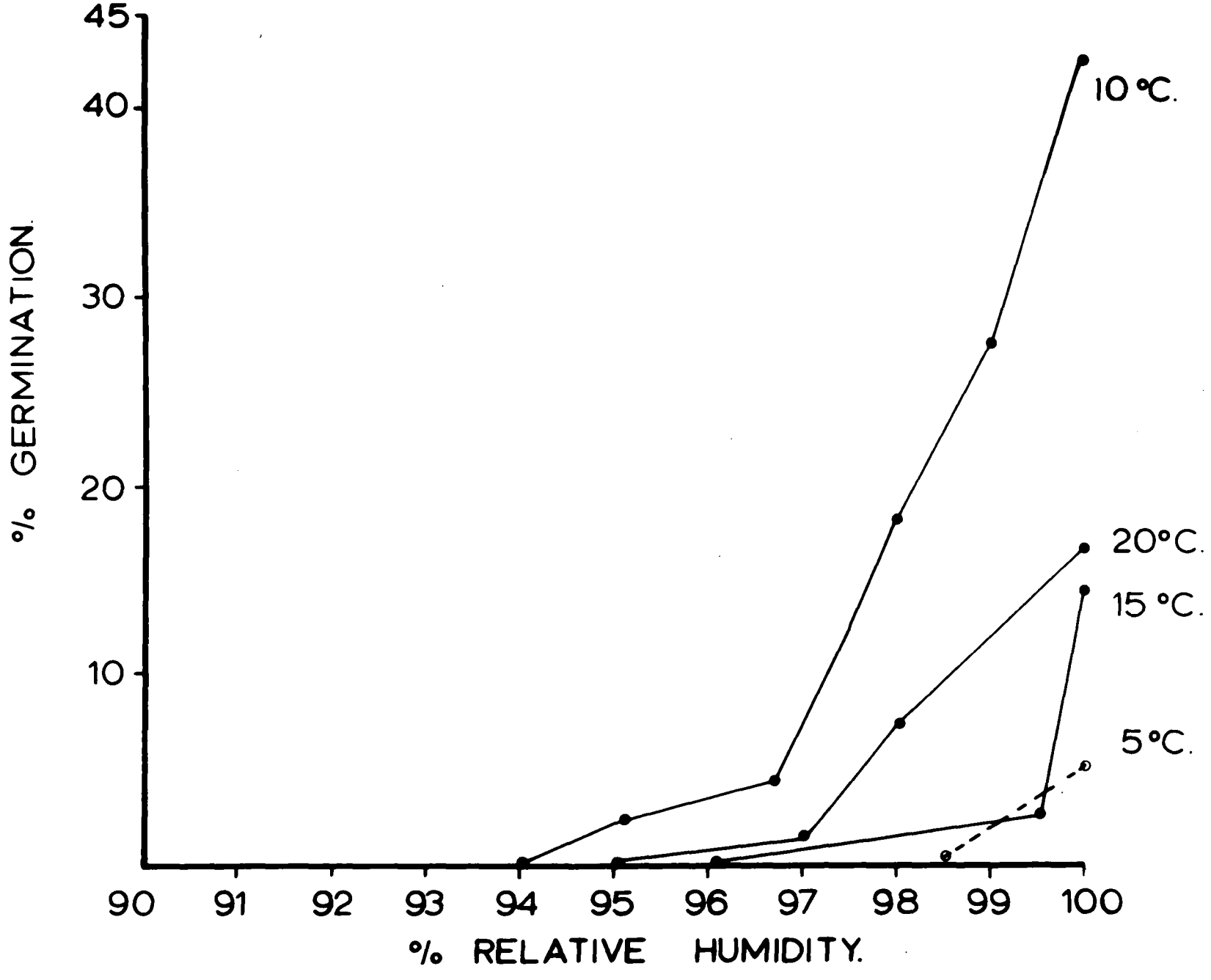


Fig. 7 P. antirrhini: Effect of humidity and temperature on germination of dry uredospores

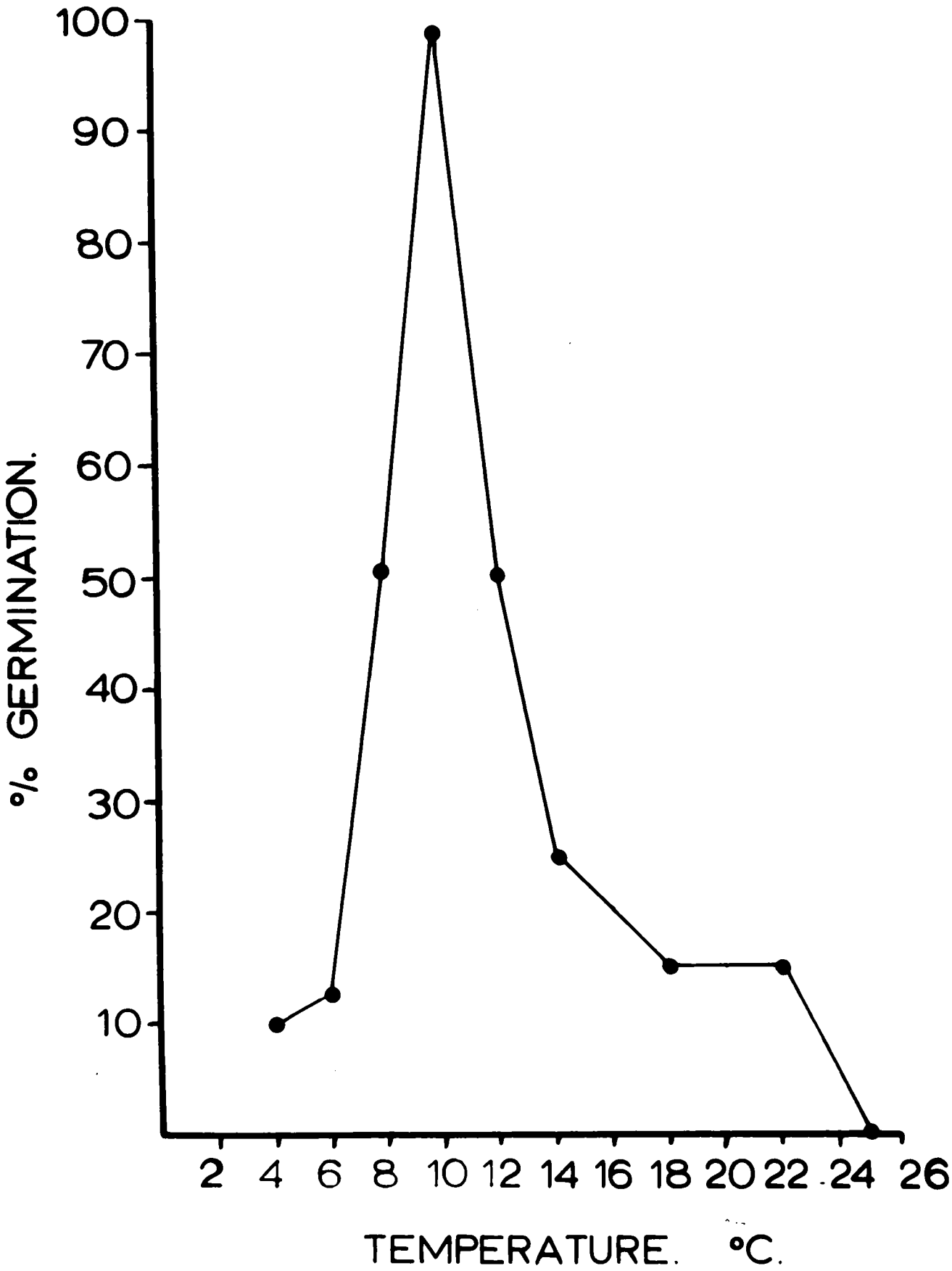


Fig. 8 P. antirrhini: Effect of temperature on uredospore germination in water

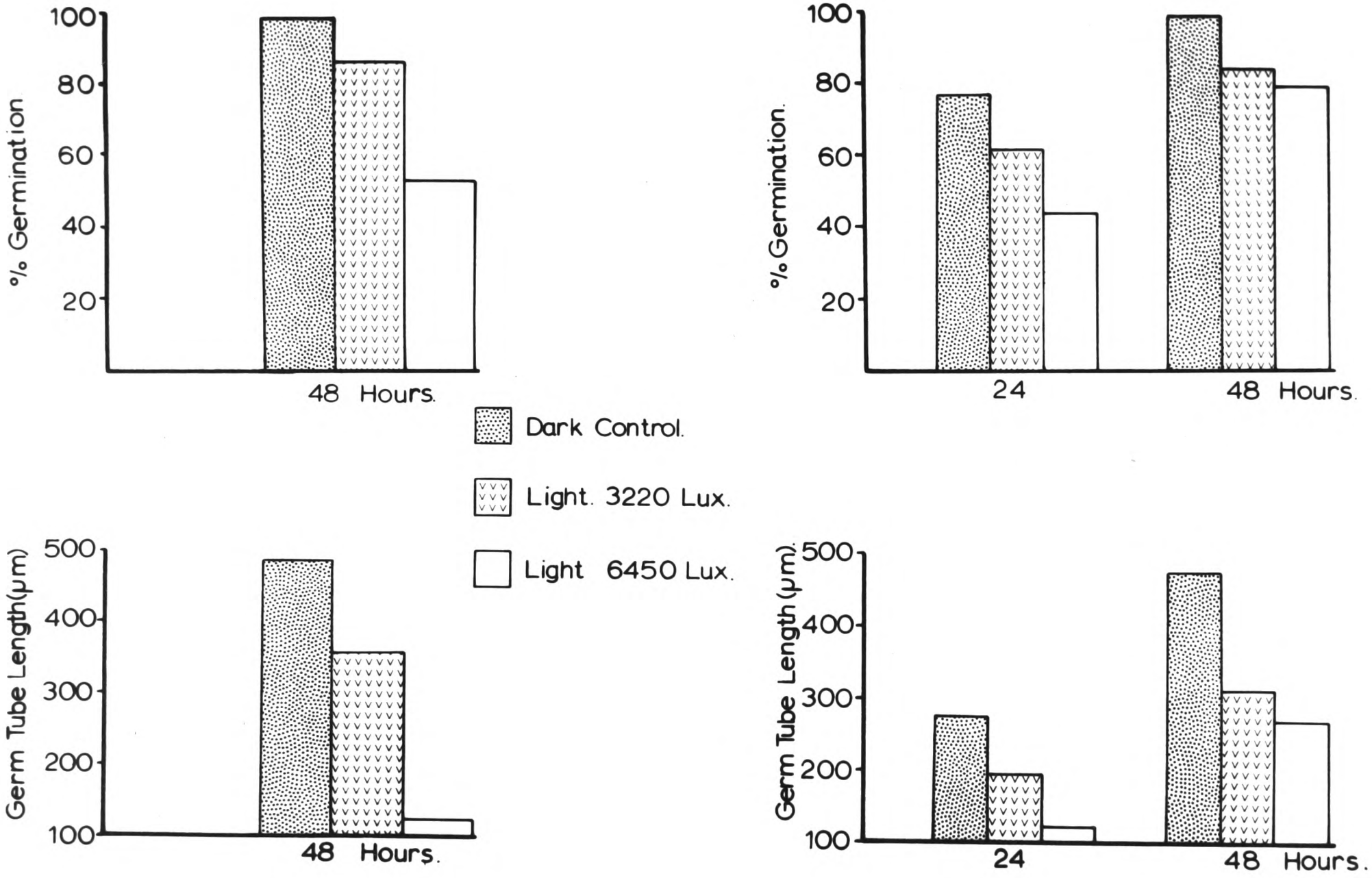


Fig. 9 P. antirrhini: Effect of light on uredospore germination

Table 11 *P. antirrhini*: Effect of spore density on spore germination *in vitro* at 10°C

Spore concentration number cm^{-2}	% Germination
100	96.3
500	95.5
1000	95.0
5000	96.1
10000	52.4

(e) SPORE AGE

Germination was maximal with spores from uredosori which had ruptured the lower epidermis of the leaf 7-10 days earlier (Table 12).

(f) RATE OF GERMINATION

Although 50% of uredospores germinated within 12 hours at 10°C, maximum germination was not recorded until 48 hours had elapsed (Fig. 10). The curves for 8°C and 12°C were similar, in that during the first 12 hours 32-35% of uredospores had germinated rising to a maximum of about 52% after 48 hours.

(g) EXOGENOUS NUTRIENTS

The presence of glucose, sucrose, or fructose did not seem to exert any significant effect ($p = .05$) on the germination or germ tube growth of uredospores (Fig. 11),

An incidental observation during these experiments was that the growth of germ tubes from uredospores took place exclusively outside the edge of the droplets placed on slides.

(2) GERMINATION OF C. CLADOSPORIOIDES

(a) EFFECT OF HUMIDITY

Germination was only observed at humidities in excess of 98% R.H. at any of the temperatures used (Fig. 12). The difference in the shape of the curves for each temperature was probably due to the impossibility of obtaining precisely the same humidities at different temperatures using the saturated salt solution method

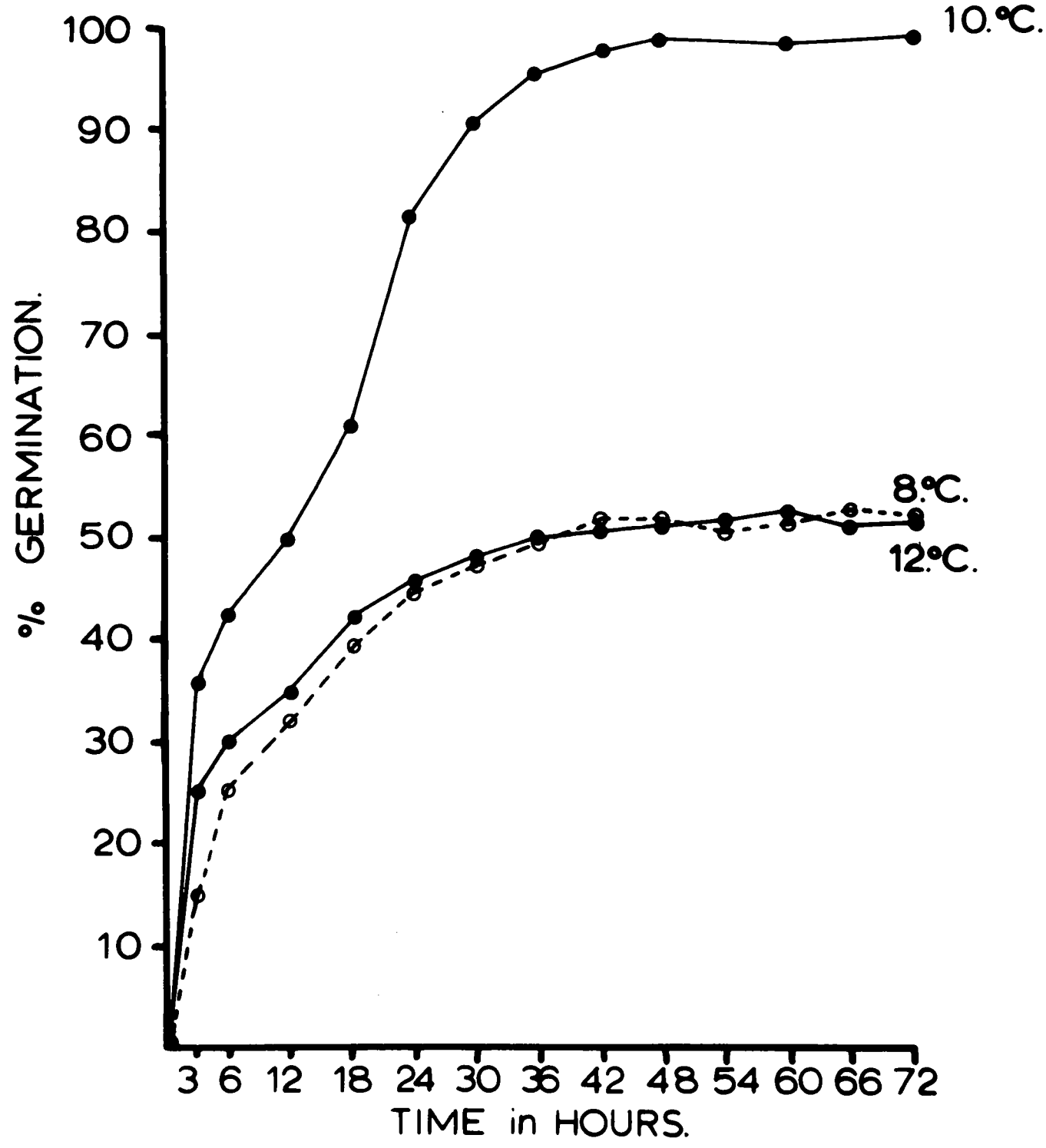
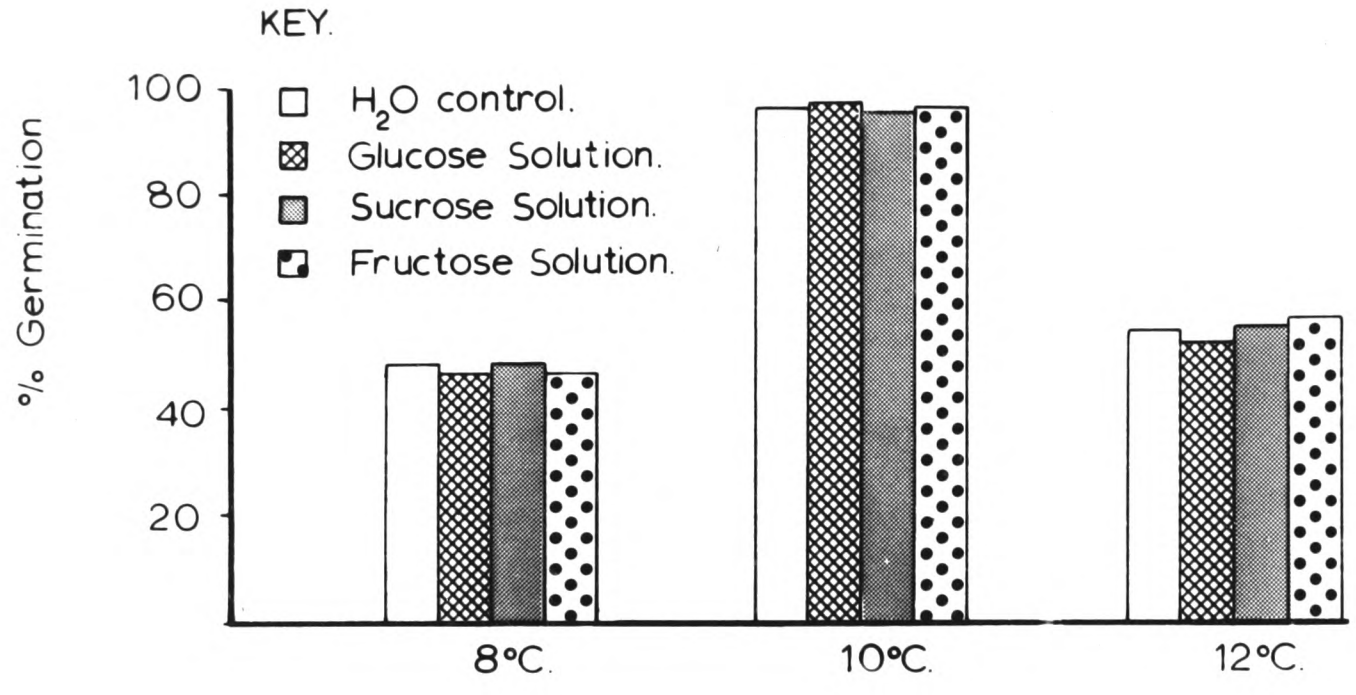


Fig. 10 **P. antirrhini:** Rate of uredospore germination at different temperatures



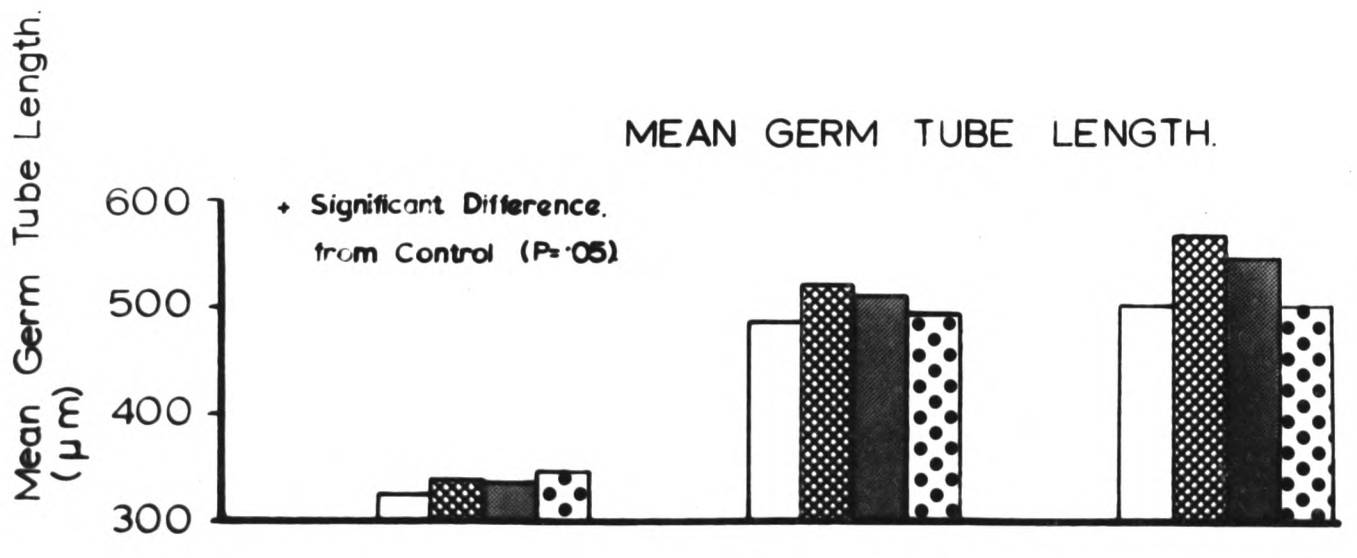


Fig. 11 P. antirrhini: Effect of exogenous sources of carbohydrates on uredospore germination

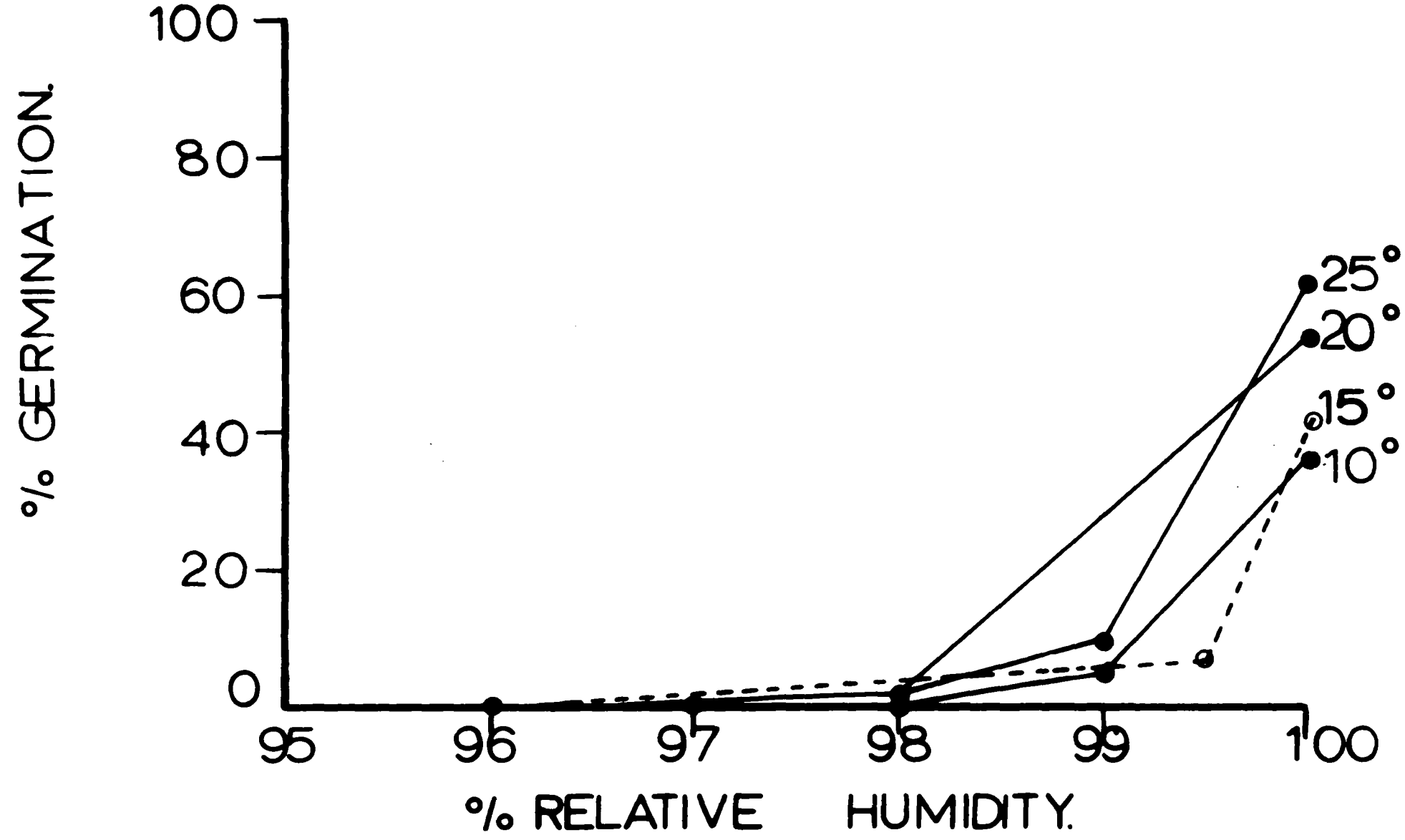


Fig. 12 C. cladosporioides: Effects of humidity and temperature on germination of dry spores

Table 12 *P. antirrhini*: Effect of age on uredospore germination *in vitro* at 10°C

Uredosorus age (Days after rupturing leaf epidermis)	% Germination of Uredospores
1	38.2
3	57.0
5	82.1
7	98.0
9	97.6
10	98.3
11	98.5
12	98.0
13	88.2
14	72.3
17	38.6
21	25.5

(O'Brien, 1948). The higher temperatures of 20 and 25°C would appear to favour the germination of C. Cladosporioides.

(b) TEMPERATURE

The optimum temperature for germination in thin films of water was shown to be 25°C (Fig. 13). This optimum was much less pronounced than that observed for P. antirrhini.

(c) LIGHT

No significant effects ($p = .05$) of light on germination or germ tube extension were observed (Fig. 14).

(d) SPORE DENSITY

Little effect due to spore concentration was observed after 48 hours incubation at 25°C (Table 13) except at very high concentrations when it became difficult to see if individual spores had germinated.

(e) RATE OF GERMINATION

The rate of germination is fairly uniform (Fig. 15) until about 42 hours have elapsed. After this time very little increase in germination occurred.

(f) EXOGENOUS NUTRIENTS

The range of carbohydrates used accelerated germination but did not alter the final percentage germination significantly ($p = .05$) (Fig. 16). However, the presence of the sugars enhanced germ tube extension significantly relative to the water control after both 24 and 48 hours incubation. Also after 48 hours incubation the median germ tube length in glucose solution was significantly ($p = .05$) greater than in the other sugar solutions.

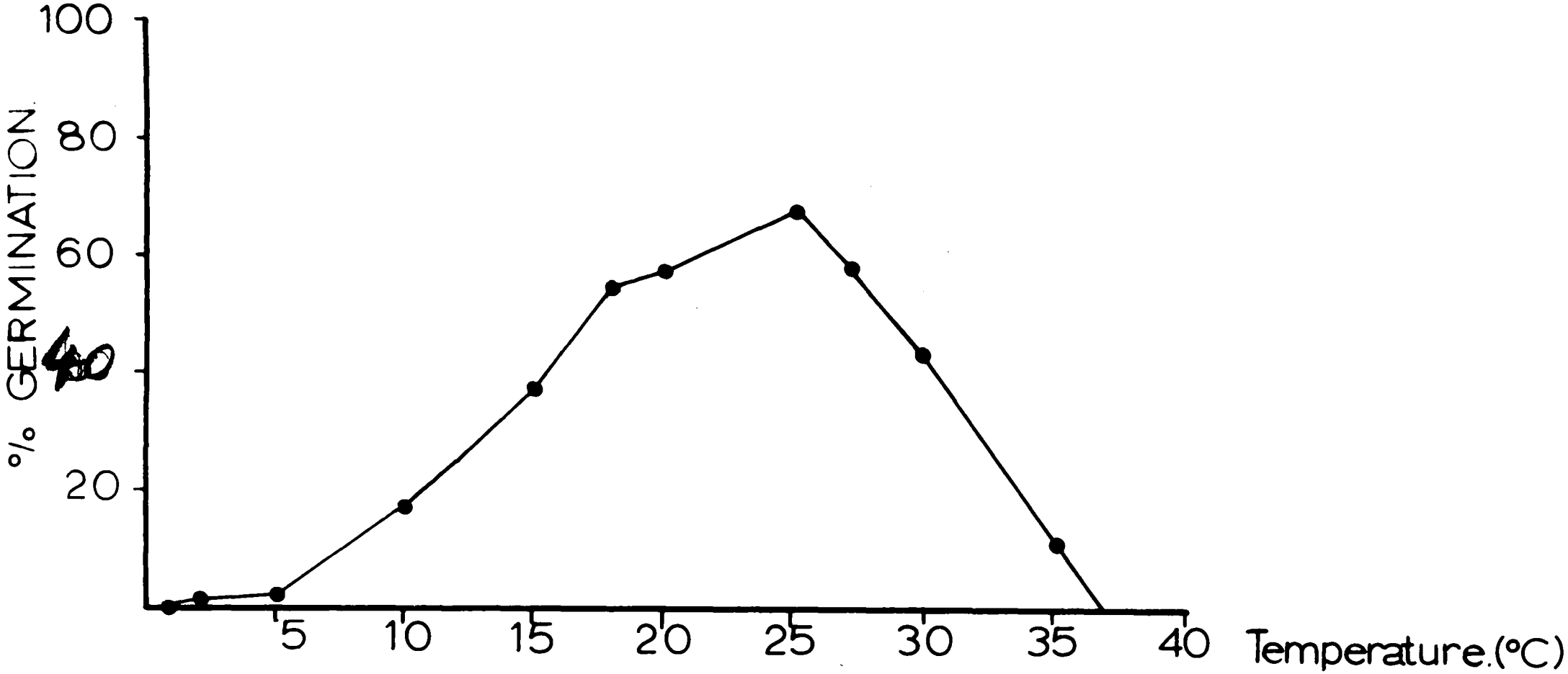
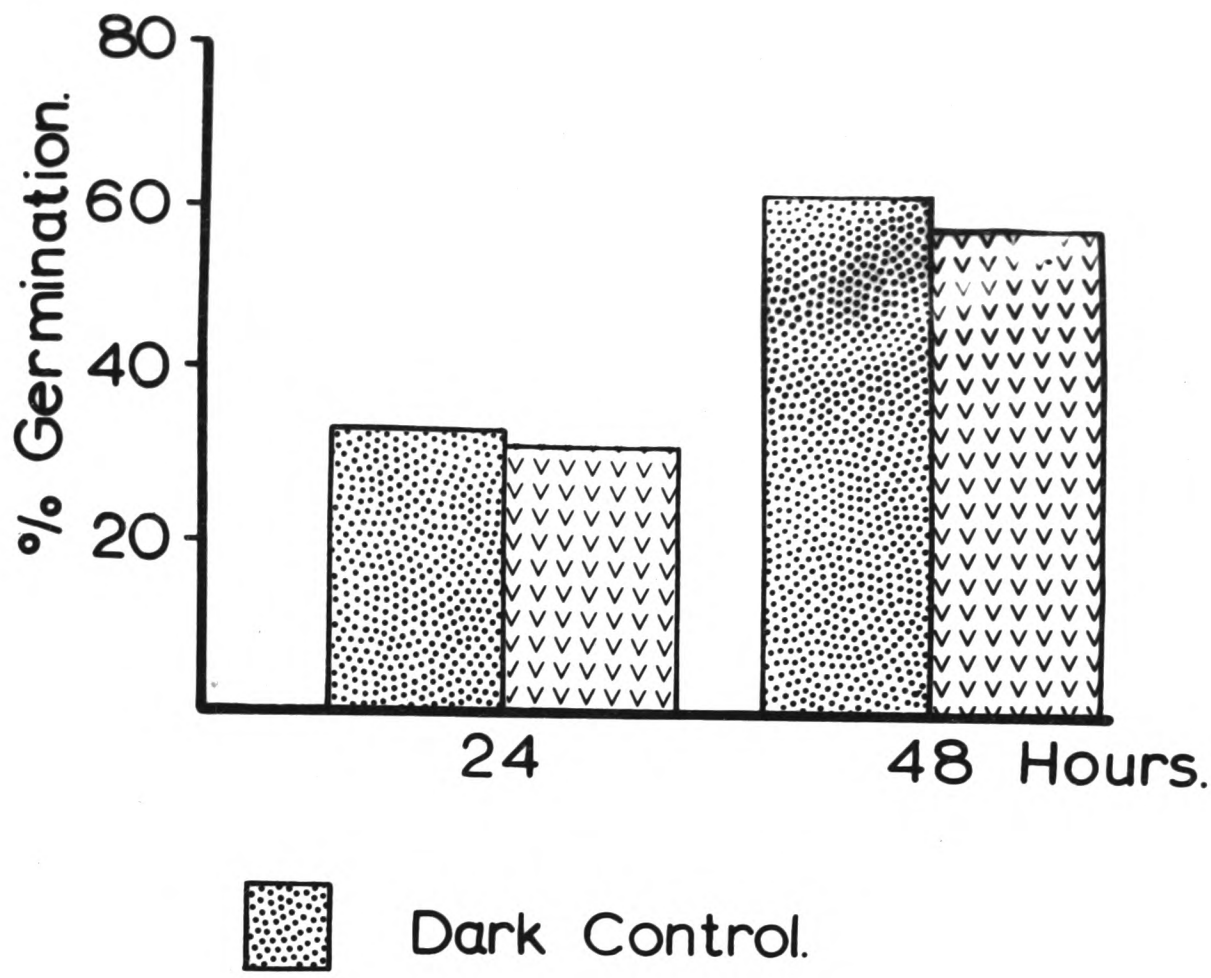
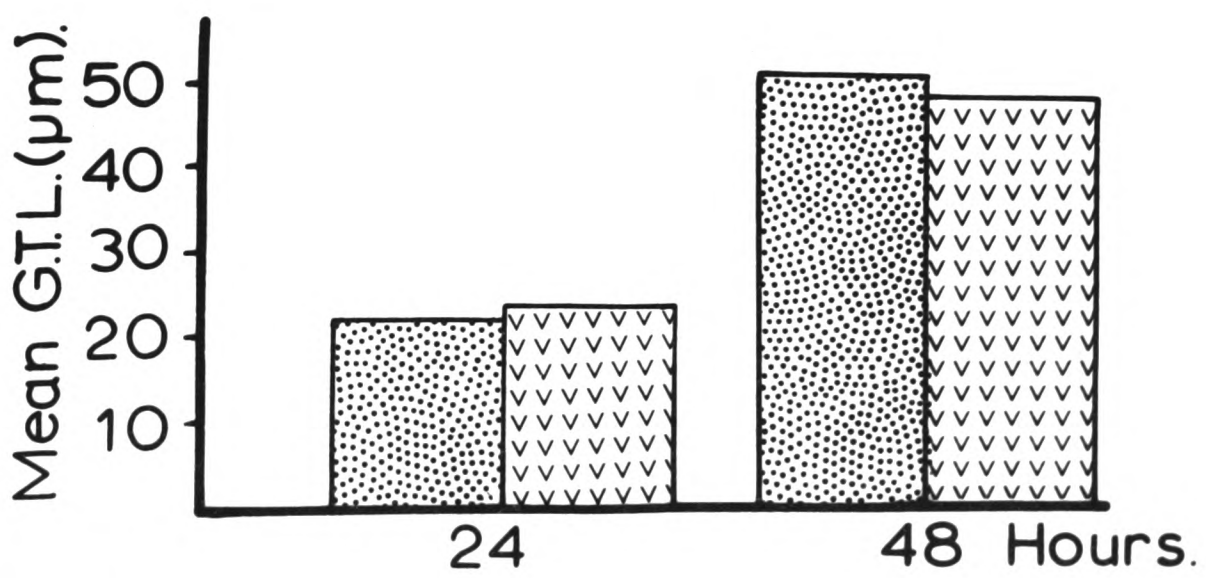


Fig. 13 C. cladosporioides: Effect of temperature on spore germination in water



G.T.L. Germ Tube Length.



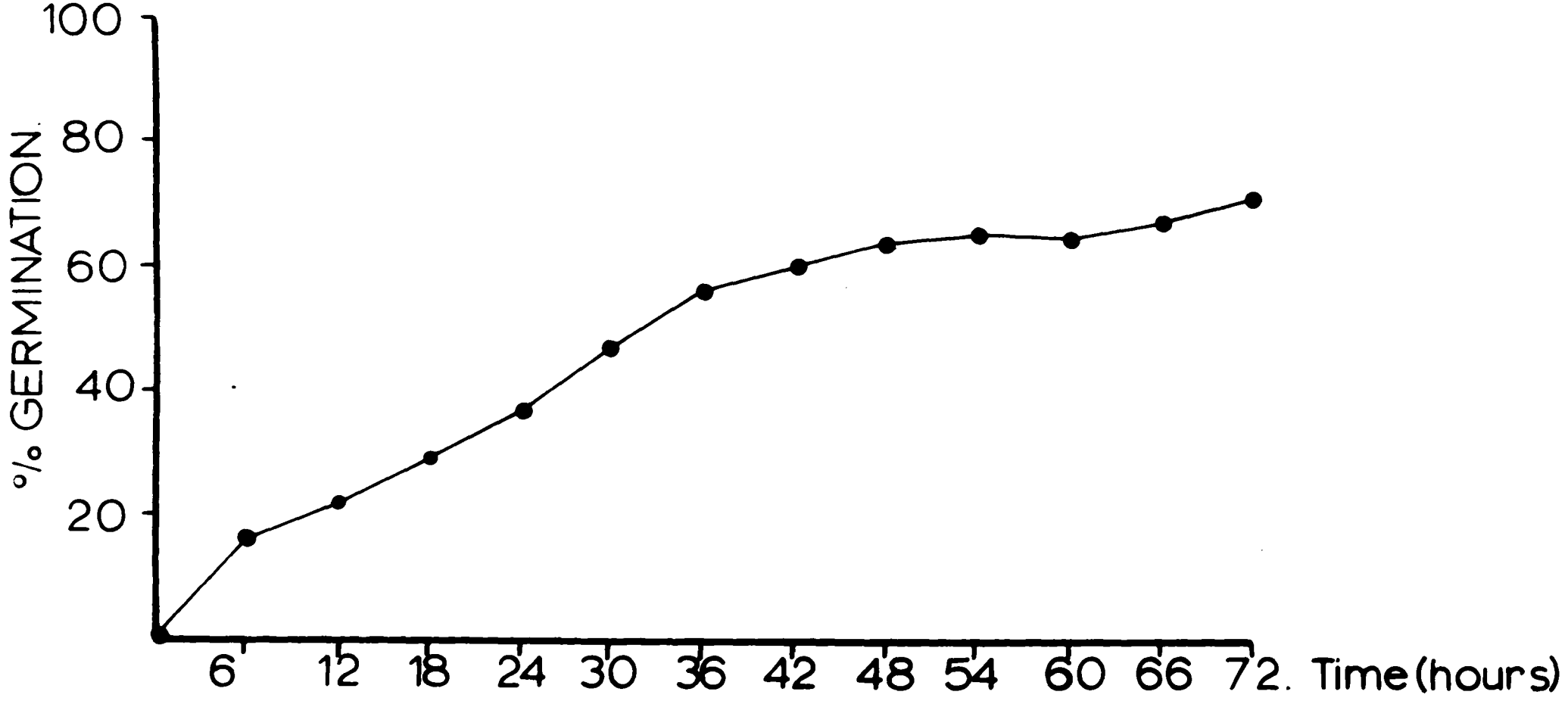


Fig. 15 C. cladosporioides: Rate of spore germination under optimum conditions

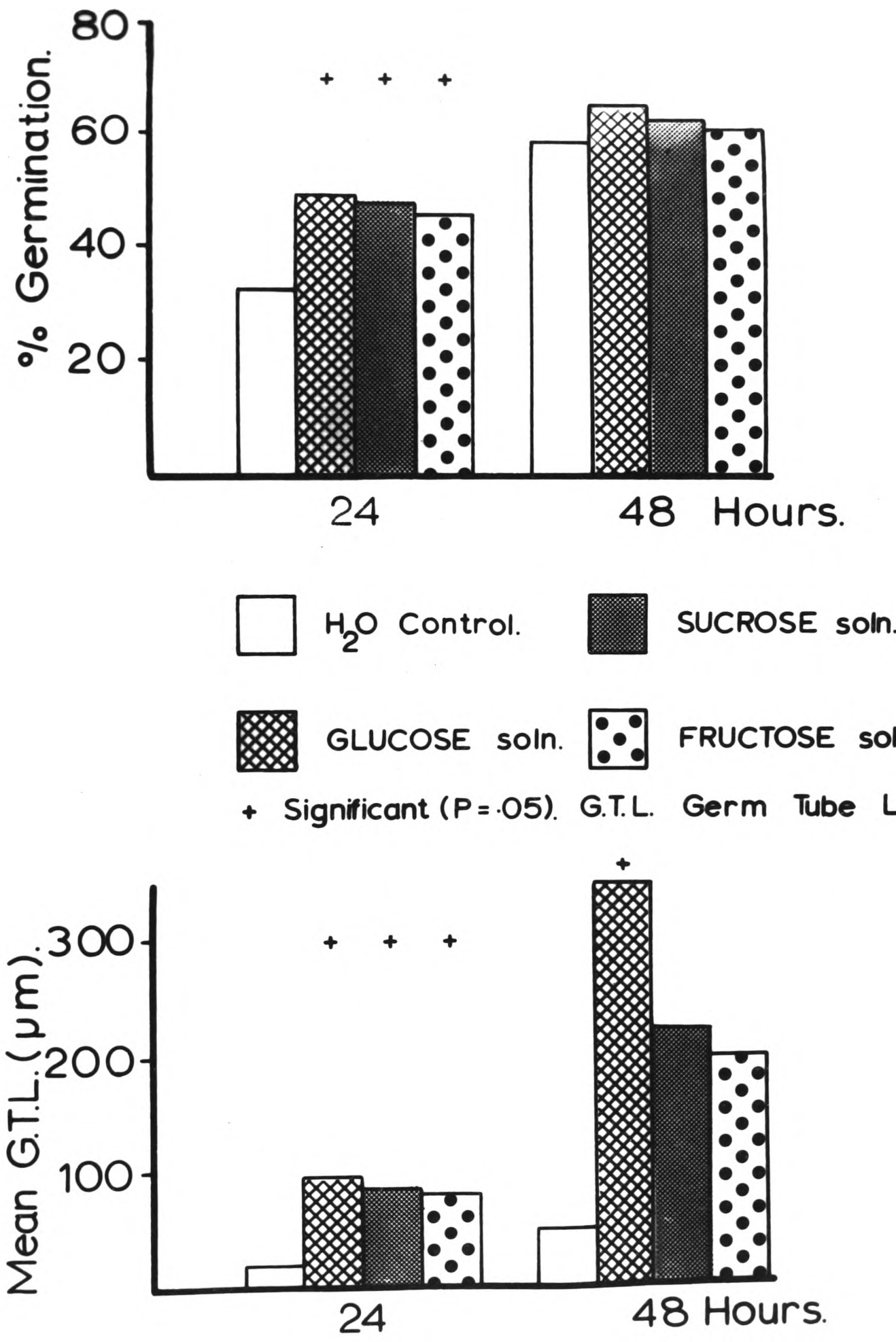


Fig. 16 C. cladosporioides: Effect of exogenous sources of carbohydrates on spore germination

Table 13 C. cladosporioides: Effect of spore density on spore germination in vitro at 25°C

Spore concentration number cm^{-2}	% Germination
100	75.5
500	76.0
1000	74.2
5000	75.0
10000	79.4
50000	77.5
100000	78.3
200000	76.0
250000	76.5

(3) GROWTH OF S. ROSEUS

The growth habit of this yeast is such that it was not possible to use the same criteria of growth as used above. The results given here refer to growth in terms of increase in cell numbers and cell size in the study of the following environmental conditions.

(a) TEMPERATURE

The highest rate of increase in turbidity and also the greatest overall turbidity recorded occurred at 18°C (Fig. 17). Practically no growth occurred at 35°C and although S. roseus did grow at 2°C the lag phase of growth was in excess of 168 hours.

(b) LIGHT

Light did not seem to exert any effect on the total volume of cells produced relative to the volume produced in the control incubated at the same temperature in darkness (Table 14).

(c) EXOGENOUS NUTRIENTS

Glucose, sucrose and fructose increased the growth of S. roseus, the effect being greatest with glucose (Table 15).

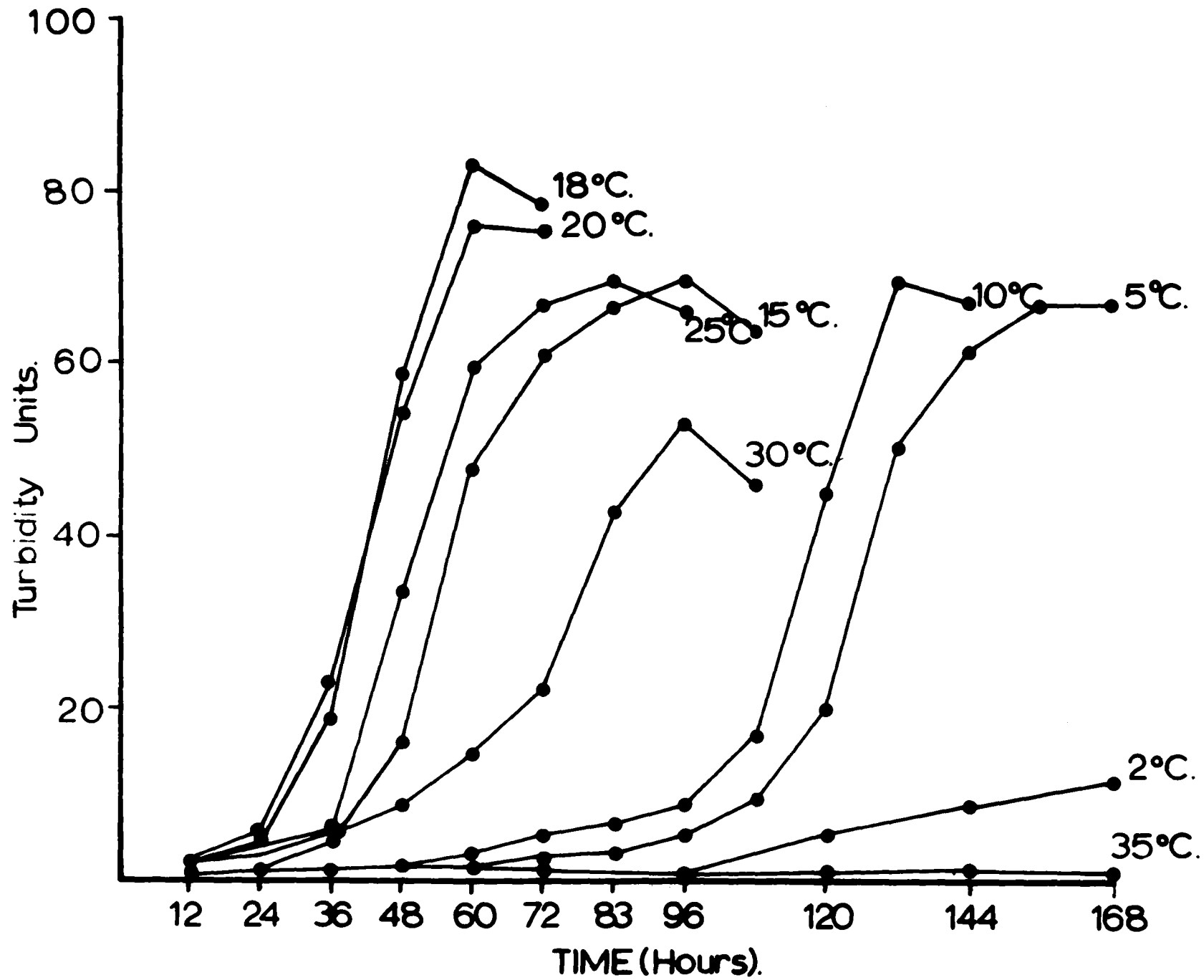


Fig. 17 S. roseus: Effect of temperature on growth measured turbimetrically

Table 14 S. roseus: Effect of light on the increase in total volume of cells in broth culture incubated for different times at 18°C

	Total volume of viable cells ($\mu m^3 \times 10^6$) ml^{-1}	
	24 hrs.	48 hrs.
Dark control	1.80	27.03
Light	1.72	28.44

Table 15 S. roseus: Increase in total volume of cells in solutions of different sugars incubated for different times at 18°C

Solution	Total volume of viable cells ($\mu m^3 \times 10^6$) ml^{-1} 24 hrs.	48 hrs.
Water control	0.22	0.23
1 mM Glucose	1.80	27.01
1 mM Sucrose	0.74	5.12
1 mM Fructose	0.95	2.59

DISCUSSION

Uredospores of *P. antirrhini* only germinated in high humidities which correlates with the observations by several workers that high humidity enhances the infection of plants and leads to an increased incidence of the rust (Doran, 1921; Green, 1934; Walker, 1954). Cochrane (1958) stated that only limited germination would occur at 100% R.H., but other workers found that high humidity would enhance uredospore germination in *Puccinia graminis* var. *tritici* Eriks. & E. Henn. Guyot (Sharp & Smith, 1957; Sharp *et al.*, 1958). Although 42.5% germination was observed at 100% R.H. in these experiments, germination in excess of 90% was achieved regularly when the spores were placed in a thin film of water. This is in agreement with Allen (1955) who observed that floating uredospores on water did improve the level of germination, but uredospores treated in this way produce a water soluble, heat stable self inhibitor of germination (Allen, 1955, 1957). However, this inhibitor is absorbed onto glass surfaces (Allen, 1955) which might account for the high percentage germination found in these tests. The increase in uredospore germination in water is due to hydration which changes the physiology of the spore (Staples & Wynn, 1965). Although free water enhances germination, in these experiments germ tubes were observed to grow away from the edge of the droplets. Burrage (1969) also described this effect and was able to show that free water could inhibit germ tube growth, although Dickinson (1949) demonstrated that germ tubes showed stimulated growth towards areas of high humidity in absence of free water.

P. antirrhini uredospores showed a very sharply defined temperature optimum at 10°C. The conflicting reports on the optimum temperature for this rust have been mentioned above (p. 72), but the results of this study agree with those of Doran (1921) for race 1 of the rust. Yap (1969) found that the uredospores of P. antirrhini germinated in the range 3-35°C, maximum germination occurring at 10 or 15°C. This isolate is believed to have been designated as race 2 and clearly shows differences in the temperature requirements for germination.

The age of the erupted uredosori was shown to exert a considerable effect on the germination potential of those spores. A similar effect has been observed for other rust spores (Cochrane, 1945; Burrage, 1969).

Maximum germination of uredospores of P. antirrhini was achieved after 48 hours incubation whereas after 24 hours only 50% of the spores had germinated (Fig. 10). This is slower than the rate recorded for P. antirrhini race 2 in which maximum germination is achieved after 18 hours at 10 to 15°C, 50% of the spores having germinated within the first 3 hours (Yap, 1969). A similar high rate of germinationoccurred inP. graminis incubated at 24°C (Sharp et al., 1958). The slow germination rate observed here may merely be a consequence of the lower metabolic rates achieved at the low optimum germination temperature.

When working with rust uredospores, some of which have been shown to produce endogenous self inhibitors of germination (Prichard, 1965), the spore concentration used in the tests may be critical. Allen (1955) found that germination was completely

inhibited when 1 mg of P. graminis uredospores were floated on a small volume of water, having a limited surface area. The results obtained for P. antirrhini showed a reduction of 44% in germination when the spore concentration was increased from 5000 to 10000 cm^{-2}. A similar reduction at these spore concentrations was observed for P. graminis (Peterson, 1959).

Continuous light was found to inhibit uredospore germination and germ tube growth. This inhibition was increased as the light intensity was increased from 3220 to 6450 Lux, supporting the findings of Sharp et al.,(1958), working on P. graminis. These workers also found that if the light intensity was increased to above 1000 ft. candles (10760 Lux), then germination was completely inhibited. This light inhibition of germination was shown to be reversible, as a subsequent dark period permitted the level of germination to rise to that observed in the 24h dark control. This form of reversible inhibition has also been observed in P. graminis (Girvan & Bloomfield, 1964) and the treatment of light followed by a dark period may even stimulate germination (McCraken & Burleigh, 1962), but no clear evidence was gained in the present experiments, on P. antirrhini, to support this suggestion.

Incubation of uredospores in sugar solutions appeared to exert no effect on germination or germ tube growth. This is hardly surprising as it has been shown that the magnitude of substrate utilisation by rust uredospores is insufficient to sustain the growth observed (Shu et al., 1956) and also uredospores are unable to utilise exogenous substrates at the rate achieved by ungerminated spores of a saprophytic fungus (Shaw, 1964).

Apart from determining the environmental conditions which are optimal for germination, the data presented here demonstrate the need for great care in the optimisation and standardisation of the procedures used for the production, storage and pregermination treatment of uredospores as all these factors affect the germination potential of the spores produced.

The data obtained for C. cladosporioides is somewhat simpler than that presented above for P. antirrhini. C. cladosporioides required a high humidity for germination (Fig. 12). This finding is supported by the work of Stott (1971) who found that humidities of nearly 100% R.H. were required for spore germination. This worker also found that the temperature range for spore germination of C. cladosporioides was 2-35°C with an optimum of 25°C which again is very similar to the data presented here (Fig. 13). No effect on germination due to spore concentration was observed when less than 250×10^3 spores were incubated in 0.05 ml. At this high value germination appeared to be reduced, but the spores were very difficult to observe. The fact that conidia of C. cladosporioides do not require exogenous nutrients for germination (Stott, 1971) may explain the lack of effect due to spore concentration. Although this study indicated that overall germination was not affected by exogenous nutrients, the presence of an external source of carbohydrate did tend to increase the rate at which germination occurred (Fig. 16), and led to a significant ($p = .05$) increase in germ tube length (Fig. 16). McBride (1970) reported that exogenous carbohydrates did enhance the germination of C. herbarum, but he may have observed the acceleration of germination in the presence of sugars. Neither of these authors stated the period of

incubation before assessment, which is important since the percentage germination of C. cladosporioides rose steadily to a maximum after 48 hours at 25°C (Fig. 15) and in C. herbarum a latent period, prior to any germination, of 6 to 12 hours at 20°C has been observed (Webster & Dix, 1960).

Light appeared to exert no effect on either germination or germ tube growth (Fig. 14).

The tests on the pink yeast S. roseus were different from those discussed above owing to the different growth habit of the yeasts compared to the filamentous fungi. The comparative turbidity data on yeasts grown at different temperatures showed that both the maximum growth rate and total growth occurred at 18°C. Derx (1930) stated that the optimum temperature for S. roseus was below 30°C and Last (1955a) found that the incubation of leaf pieces at 22.5°C gave higher counts using the sporefall method than incubation at 10°C. These would infer that the optimum temperature for the growth of S. roseus was above the psychrophilic temperatures which have been shown to be optimal for the isolation of many yeasts from tree leaves (Beech & Davenport, 1971) and support the observations recorded here.

Light did not seem to have any discernable effects on the growth of S. roseus in a glucose solution. This would be expected as it has been shown that the carotinoid present in the yeast protect it from photodynamic effects (Maxwell et al., 1966).

The presence of carbohydrates in solution did give rise to significant increases in growth, as measured by total calculated volume, over the control culture in water. The growth was greatest in glucose, then sucrose, then fructose. This is not the result that one might have expected as Slator (1906, 1908) has shown that glucose and fructose are fermented at the same rate. Also, yeasts grown on maltose can respire immediately with glucose and fructose, but not sucrose (Tustanoff & Bartley, 1964). However, Menzinsky (1943) showed that Slator's results only applied if the concentrations of sugars were fairly high (1-10% for glucose and 2-8% fructose). As the concentrations of sugars used in this experiment were considerably lower than these levels, then the findings of Menzinsky (1943) may apply in this case and so it would be reasonable to expect glucose to be fermented more rapidly than fructose. In the case of sucrose, although S. roseus grown on maltose cannot respire immediately with sucrose, after a lag phase the rate of sucrose hydrolysis may rise as high as 300 times the rate of fermentation by the yeast (Demis et al., 1954) which means that glucose as well as fructose would be freely available, again supporting the arguments of Menzinsky (1943) quoted above. These observations show that all three carbohydrates commonly found in the leachates from leaves (Nyvist, 1963; McBride, 1970; Purnell, 1971) are utilised by S. roseus even though the relative rates of utilisation are different.

The in vitro tests using these organisms revealed the need for standardisation of the conditions used to grow these organisms prior to experimental use. The data on the effects of environmental conditions could be used as the basis for determining the regimes to

be used in further experiments to examine interactions between plants and microbes and also between the different micro-organisms which exist on the leaf surface.

SECTION 2 THE PLANT COMPONENT

SECTION 2: THE PLANT COMPONENT

INTRODUCTION

One of the most essential criteria for the selection of a plant/pathogen system for the study of plant microbe inter-relationships is that both components should be amenable to laboratory culture. For this reason A. majus was chosen as the plant component since its specific obligate pathogen P. antirrhini would infect plants grown in greenhouse conditions and the incubation period was short. Furthermore, the pathogen was relatively easy to obtain. This apart, A. majus is useful as both rust resistant and susceptible horticultural cultivars are available and the host has the advantage that it can be grown throughout the year merely by supplementing the available daylight (Petersen, 1957). The leaves produced by these plants are reasonably uniform and are of a size which allows the use of entire leaves for many purposes.

In this study the development of plant and leaves was observed to enablea Plastochron Index (Erickson & Michelini, 1957) to be established so that leaves of similar physiologic ages could be used in experiments. The anatomy and nutrient status of the leaf surface were investigated in order to obtain some information on the environment in the phylloplane of these plants. Finally a series of experiments is described concerning the establishment of a technique for growing antirrhinum plants under aseptic conditions. Although this technique has as yet not been used in full scale experiments, it is presented here to show the types of difficulties encountered.

Nevertheless, investigations of this type are necessary so as to obtain a better understanding of the processes of colonisation of actively growing plants by micro-organisms and micro-organism interactions which might take place on plants.

REVIEW OF LITERATURE

(a) A. MAJUS

Antirrhinum majus appears to have received limited attention hitherto. A taxonomic description may be obtained from Flora Europea (Tutin et al., 1963), which reveals that the snapdragon is a member of the Scrophulariaceae. It is a perennial herb growing, in either erect or prostrate form, to a height of 2 m. It has linear to oval leaves (10-70 x 1-25 mm), a pink or purple corolla, and an oblong capsule which may be glandular, pubescent or glabrous. In Europe, it is native to the Mediterranean area extending from south west Europe to Sicily. It is cultivated for ornamental purposes and is naturalised or cultivated on a field scale throughout Europe from Turkey to the north of Germany and the United Kingdom. Four overlapping subspecies are listed. Plants which have naturalised after escape from cultivation can be assigned, in the Mediterranean area, to the subspecies tortuosum, or elsewhere to the subspecies majus, but hybridisation which is both common and widespread can lead to taxonomic difficulties. The subspecies majus which accounts for the majority of plants cultivated in the U.K. exhibits the following characteristics. The stem may grow to a height of 1.2 m and bears elliptic-lanceolate to linear oblong shaped leaves (30-70 x 5-70 mm). The inflorescence is glandular to pubescent with a purplish pink corolla (height 30-45 mm) and yellow palate. This subspecies is native in the Pyrennean area but is widely naturalised elsewhere.

In the United Kingdom naturalised snapdragons are distributed mainly in the following areas: Bristol; South coast of England; Thames valley; East midlands; Welsh borders; North Wales coast; North west England; Lothian area of Scotland; East coast of Ireland. These areas are characterised by low altitude (mainly below 150 m), total rainfall of less than 1030 mm and mean temperatures in the range 4-17.5^{o}C (Perring & Walters, 1962).

Even though naturalised plants are found in Britain the majority of snapdragon plants are cultivated. These plants are important horticulturally, especially for use as bedding plants, as flowering spikes are long lived and available in a variety of colours. A measure of their importance may be gained from the estimate of 17×10^{9} plants grown annually (Chittenden, 1934). Although plantings were reduced during the 1930's owing to the epidemic of P. antirrhini (Green, 1934), experiments to produce resistant cultivars were done (Green, 1937). Resistant cultivars bearing attractive flowering spikes were produced by the 1940's (Green, 1941) allowing large numbers of antirrhinums to be grown again. Today, although many of the plants grown are resistant to the rust disease, the older susceptible varieties are grown in many areas probably due to the relatively lower cost of these seeds. This fact is especially important in public gardens where very large numbers of plants are grown (Brooks, personal communication).

(b) NATURE AND ENVIRONMENT OF THE LEAF SURFACE

Although the description of leaves of A. majus given in Flora Europea may be adequate for the purposes of identification, it is insufficiently detailed for a critical study on the phylloplane of this plant. A more detailed description could be obtained by following the classification of the architecture of dicotyledenous leaves proposed by Hickey (1973). After the categorisation of such features of the whole leaf and of its base and apex, the leaves are separated into a number of classes depending on the course of the principal venation. The identification of the order of venation which is fundamental to the application of the classification is determined by the size of a vein, its point of origin, and to a lesser extent by its behaviour in relation to that of the other orders. The classification concludes by describing features of the areoles (the smallest areas of leaf tissue surrounded by veins which form a contiguous field over most of the leaf).

Although such a classification is very rigorous and useful in taxonomic studies, it would reveal very little of the fine detail of the surface of the leaf and the environment prevailing there. A review of the work on the surface characteristics of leaves has been done (Martin & Juniper, 1970). In this they describe the cuticle as the layer overlying leaf epidermal cells as a continuous membrane which also, in a thinner form, lines the substomatal cavities. The physiological functions of the cuticle are described as including the trapping and absorption of water (Amsden & Lewins, 1966); the protection of the leaf against mechanical damage (Schopmeyer, 1961)

including frost damage (Hall & Jones, 1961), the control of water loss (Horrocks, 1964), guttation (Ivanhoff, 1963), nutrient loss (Tukey & Morgan, 1964) and carbon dioxide exchange (Holmgren et al., 1965). The ability of the cuticle to perform these functions is due to its multilayered structure. Wax is an important component of the cuticle which may be present as a superficial layer as well as being embedded in the cutin which is regarded as the chief structural component of the membrane (Martin & Juniper, 1970). The chemical characteristics of the leaf surface are normally governed by the surface wax layer (Holloway, 1971). Wax formation takes place at an early stage of leaf growth and usually continues throughout the period of leaf expansion (Richmond & Martin, 1959). However, no relationship exists between the waxiness of the surface and thickness of the cuticular membrane (Holloway & Baker, 1970). These plant surface waxes are complex mixtures which normally comprise various classes of long chained aliphatic compounds (Eglinton & Hamilton, 1967), but in some cases appreciable amounts of cyclic compounds such as Ursolic acid may also be present (Holloway, 1971).

The physical properties of leaf surfaces are markedly influenced by their roughness. This may be caused by venation, shape of the epidermal cells and the cuticle surface, the presence or absence of trichomes and surface wax. When surface waxes are present their shape and distribution contribute to the surface roughness (Holloway, 1971).

The surfaces of leaves show considerable differences in their wettability which ranges from completely wettable to highly water repellent (Amsden & Lewins, 1966; Holloway, 1969). There is no definite correlation between the hydrophobic properties of a leaf

surface and the amount of surface wax present (Silva Fernandes, 1965), but the occurrence and distribution of wax structures may be correlated with this property (Thrower et al., 1965). Water repellancy is greatest when the wax has a rough surface in the form of projecting rods, or a crystalline or semi-crystalline structure (Juniper, 1959). The differences in leaf wettability are not wholly accounted for by the occurrence of different waxes or in the chemical or hydrophobic properties of the isolated surface waxes (Holloway, 1969).

The wettable nature of a leaf is related not only to the moisture conditions prevailing on the surface, but also to the phenomenon of the susceptibility of the leaf to nutrient leaching (Fogg, 1947). Young leaves are hydrophobic and wetted with difficulty (Linskens, 1952) and are less susceptible to leaching than other leaves (Cholodny, 1932). The leaching of substances from plants appears to occur in a large number of species (Arens, 1934; Tukey & Morgan, 1964). However, various plants exhibit different susceptibilities to leaching. The leaves of pear, apple, gooseberry and plum were leached more easily than blackcurrants. The resistance to leaching may differ even between cultivars of the same species (Wallace, 1930). Differences in susceptibility to leaching have been observed not only between species but also between individual leaves of the same plant (Schoch, 1955). As mentioned above young actively growing leaves are relatively immune to the loss of nutrients, but as the leaf matures its susceptibility to leaching increases reaching a peak at senescence (Arens, 1934; Lausberg, 1935; Stenlid, 1958). This phenomenon may be illustrated by studying the leaching of potassium from leaves. Young leaves were

observed to lose up to 5% of their initial potassium with 24 hours (Tukey et al., 1958), but in mature leaves the loss may be greater than 80% (Wallace, 1930).

Despite the large quantities of inorganic substances which may be leached from leaves, organic substances, principally carbohydrates, account for the major quantities of leached materials. Dalbro (1956) calculated that the loss of carbohydrates may be as great as 800 Kg Hectare^{-1} year^{-1} from apple trees. In another study it was found that up to 5% of the dry weight equivalent, mainly in the form of carbohydrates, could be leached from young bean leaves during a 24 hour period (Tukey et al., 1958). A great diversity of substances including amino acids, growth substances, alkaloids and phenolics may be leached from plants in addition to carbohydrates and simple salts (Morgan & Tukey, 1964).

Another factor which affects the environment on the leaf surface is the microclimate which prevails close to this surface. Surrounding each leaf is a thin boundary layer of air influenced by the leaf surface (Sutton, 1953), the thickness of which has been estimated using Schlieren optics (Yabuki et al., 1970). Measurements of various components of this microclimate have been attempted. Temperature has been measured using fine thermocouples, which revealed that the temperature varied in different areas of the leaf (Waggoner & Shaw, 1952). The effects of the physical nature of the leaf surface on the selective transmission of certain wavelengths of radiation has been observed by Billings & Morris (1951) who found that differences in leaf surfaces affected the temperature of the leaf. Up to the present time humidity has usually been measured on

a fairly gross scale within a crop (Burrage, 1971), although the humidity close to dock leaves has been measured in laboratory conditions (Ramsay et al., 1938). Further development of miniature probes for measuring humidity (Monteith & Owen, 1958; Cotton, 1969) may enable finer measurements to be obtained. A major source of moisture on the leaf surface is dew, the formation of which has been studied in detail (Burrage, 1969; Clifford, 1973). The influence of windspeed on the boundary layer in relation to the physical characteristics of the leaf has also been investigated (Wooley, 1964). The observations of these conditions is important in phylloplane studies as complex interactions between host, parasite and the environment have been observed during the infection of wheat by P. graminis (Burrage, 1970). There is no reason to suppose that this example was an isolated case of such interactions occurring between micro-organisms and the environment at the leaf surface.

There are indications that the physical and nutritional environment prevailing at the leaf surface is influenced by the age of the leaf (Stenlid, 1958; Martin & Juniper, 1970). Many characteristics of the leaf change as it ages. The examination of morphological characters such as wet or dry weight, cell size, cell numbers and physiological characters such as the relative amounts of nucleic acids and chlorophyll content use either destructive techniques or procedures which are rather too complex and lengthy for continual routine use. When the leaves are required for further work some non-destructive method of characterising leaf age has to be sought. The growth of leaves in terms of length or area would seem to be suitable for this purpose, but problems of measurement and interpretation may occur. The ultimate size of a leaf is

determined by the number of primordial cells, the rate of cell division, the duration of the phase of cell division and the size of the individual mature cells (Gregory, 1956). The interplay of these factors may cause complications in the estimate of leaf age and growth. An additional factor is that the growth of leaves is not a linear function of time. In dicotyledons the main increase in size of the leaf is due to cell expansion as cell division more or less ceases as the buds unfold and the subsequent rate of increase in size is temperature dependant (Gregory, 1956). However, it is possible to express the leaf shape in quantitative terms and with the measurement of appropriate lengths and angular characteristics, the changing shape of the individual leaf or successive leaves can be expressed as a function of time (Ashby, 1948).

A long and involved article on the analysis of growth in quantitative terms was given by Richards (1969). He stated two theoretical principles which are fundamental to growth studies. The cell multiplication in higher plants is an expression of growth and so long as cells remain meristematic and continue to divide at uniform intervals, the cell number must obey the exponential law of increase. This exponential growth evidently cannot continue indefinitely for it would lead eventually to enormous numbers of cells or gigantic plant size. Thus, although this law may hold initially, at a later time period the increase becomes progressively smaller than that predicted by this law and when the incremental size reduces to zero, growth has ceased. The second principle is not quantitatively defined, but is descriptive, stating in general terms that where growth is progressively restricted by loss of meristematic activity and the processes of maturation and

differentiation, the increment per unit time first increases to a maximum and then declines to zero. He reviews in detail the properties of curves and the attempts that have been made to define the grand curve of growth proposed by Sachs (1874) and account for it quantitatively in terms of broad physiologic generalisation.

Some of the research dealing with the influence of leaf age on its physiological characteristics has used a simplified approach to the problem by taking advantage of the fact that the leaves present on a shoot at any particular time constitute an age series. This makes the comparison of leaves of different ages an easy matter and also reduced sampling error (Hover & Gustafson, 1926). When using plants which show progressive or sequential leaf senescence (Wareing & Phillips, 1970), the leaves on any stem would constitute an age series, but the use of the leaves present on a stem at one time to deduce the history of single leaves at successive stages has been criticised severely (Richards, 1934). He pointed out that even at comparable ages successive leaves constituted a series of inherently different physiological structures. This is due to the different patterns of nutrient translocation which occur as the leaves become further removed from the growing point (Kriedemann, 1968). Richards (1934) recommended the use of corresponding leaves on replicate plants even though the sampling errors involved were larger. However, even this approach may involve problems in interpretation, for unless genetically uniform plants are used under exactly controlled conditions, variability may be so great that plants of the same chronological age may have reached quite different stages of development, while plants which are morphologically similar may be of quite different chronological

ages. Under such conditions significant relationships between plants or leaves may be obscured (Michelini, 1958).

One method of linking the physiological development of a plant to time was the proposed use of a plastochron index for the study of vegetative shoot development in Xanthium italicum Moretti (Erickson & Michelini, 1957). The term plastochron was proposed by Askenasy (1880) to delimit the time interval between the formation of two successive internode cells of Nitella flexilis Agardh. More broadly it can be defined as the time interval between the initiation of any two successive leaves, or between these leaves at corresponding stages of development which can be used as a reference stage. Erickson and Michelini (1957) measured the lengths of successive leaves of Xanthium plants and used the logarithm of leaf length plotted against time to produce a family of curves which showed clearly that the time intervals between successive leaves at the same stage of development were equal. This fact combined with the parallel nature of the curves over a period of several days and the properties of the logged values of any growth characteristic (Fisher, 1921), enabled them to devise an index which specified precisely the state of the vegetative shoot within an error of only a few hours. A similar related index of leaf development was also devised. A detailed 10 year study of the morphological patterns and physiological aspects of development of intact leaves of X. italicum in the normal situation has used the Leaf Plastochron Index to define the developmental status of leaves from initiation to their eventual death with a degree of statistical accuracy which would have been impossible without the use of such an index (Maksymowych, 1973).

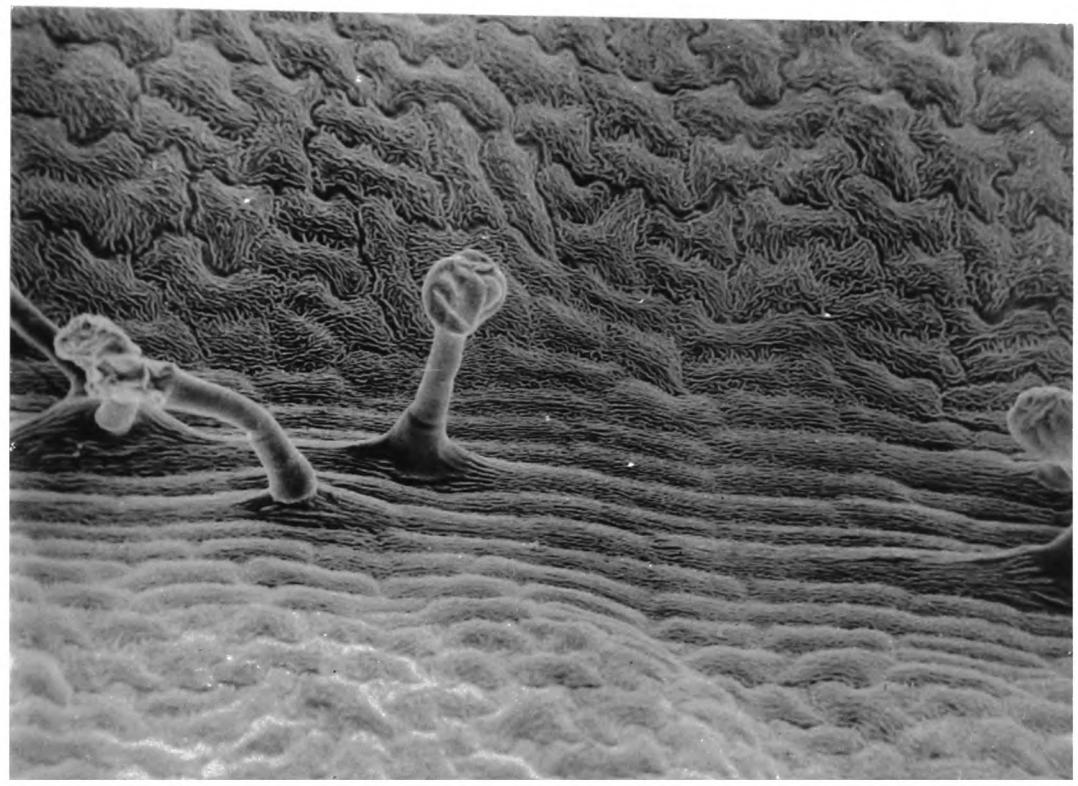
DESCRIPTIVE

PHYSICAL NATURE OF THE LEAF SURFACE OF A. MAJUS

A brief survey of the surface anatomy of antirrhinum leaves was conducted using a Scanning Electron Microscope (SEM) (Cambridge Instrument Stereoscan 2a), supplemented by light microscopy in one case. Leaves were examined both before the exponential phase of growth and at a later stage when the rate of expansion began to decrease and the leaf became mature. These leaves had Leaf Plastochron Indices (LPI) of -0.5 and 1.5 respectively (p. 116). The leaves studied were taken from greenhouse grown plants. Squares of leaf tissue (5 x 5 mm) were removed from the leaves using a scalpel and fixed to metal SEM stubs using Silver Dag (Acheson Chemical Corporation) and then coated, under vacuum, with gold palladium alloy, before examination using the SEM.

A series of electron micrographs are presented here to illustrate the main surface features of antirrhinum leaves of both varieties at the developmental stages indicated above. The young leaves (LPI -0.5) of A. Nanum (Plate 3) showed a differentiated surface of laminar and midrib complete with glandular hairs. The number of stomata was markedly lower on the adaxial surface (Plate 4) than the abaxial surface (Plate 5). The surface of the cuticle on both surfaces of the leaves showed marked striations (Plates 4, 5). Similar characteristics were observed on the young leaves of A. Fi hybrid (Plates 6, 7).

Plate 3. Scanning electron micrograph of young leaf of A. Nanum (LPI -0.5) showing midrib zone with glandular hairs (x 180)



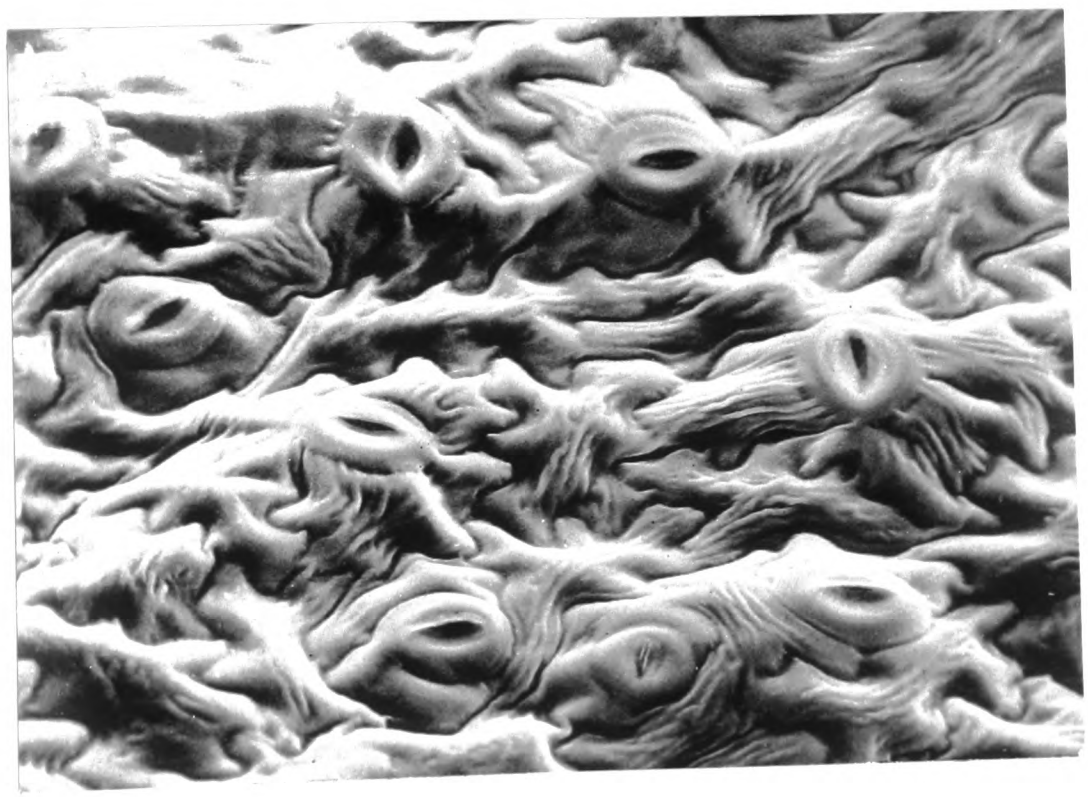
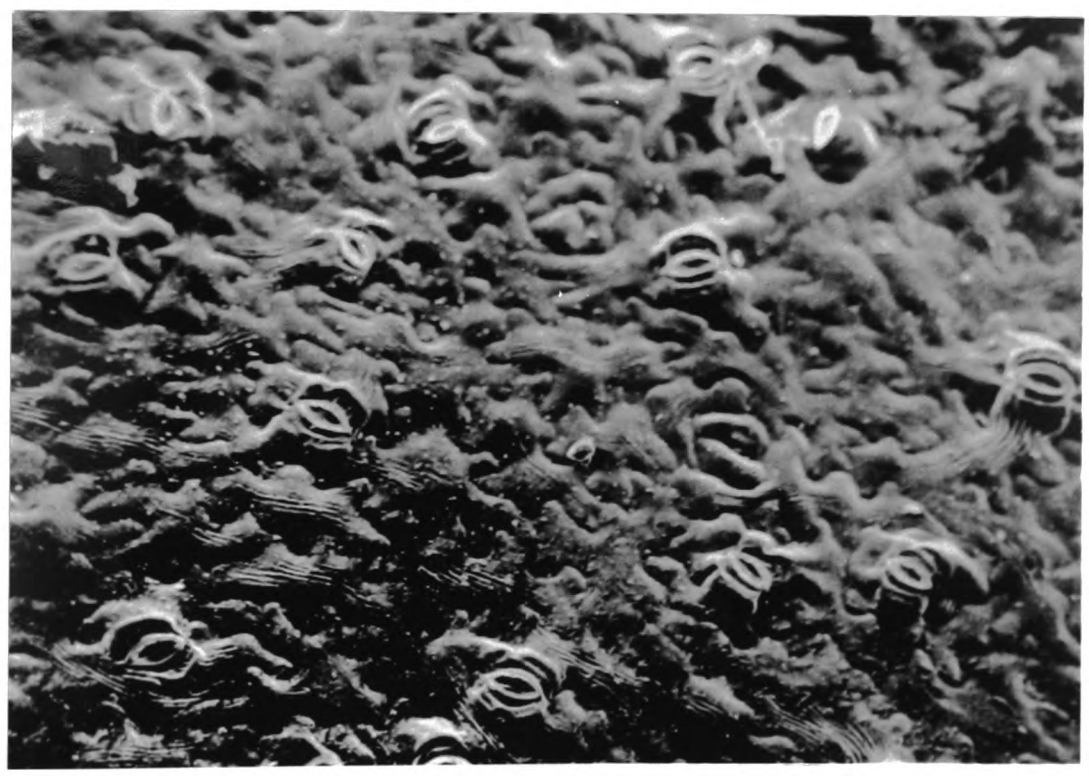


Plate 6. Scanning electron micrograph of the upper surface of young leaf (LPI -0.5) of A. Fi hybrid, showing cuticular striations (note the complete absence of stomata) (x 446)

Plate 7. Scanning electron micrograph of the lower surface of young leaf (LPI -0.5) of A. Fi hybrid showing cuticular striations and frequency of stomata (x 187)

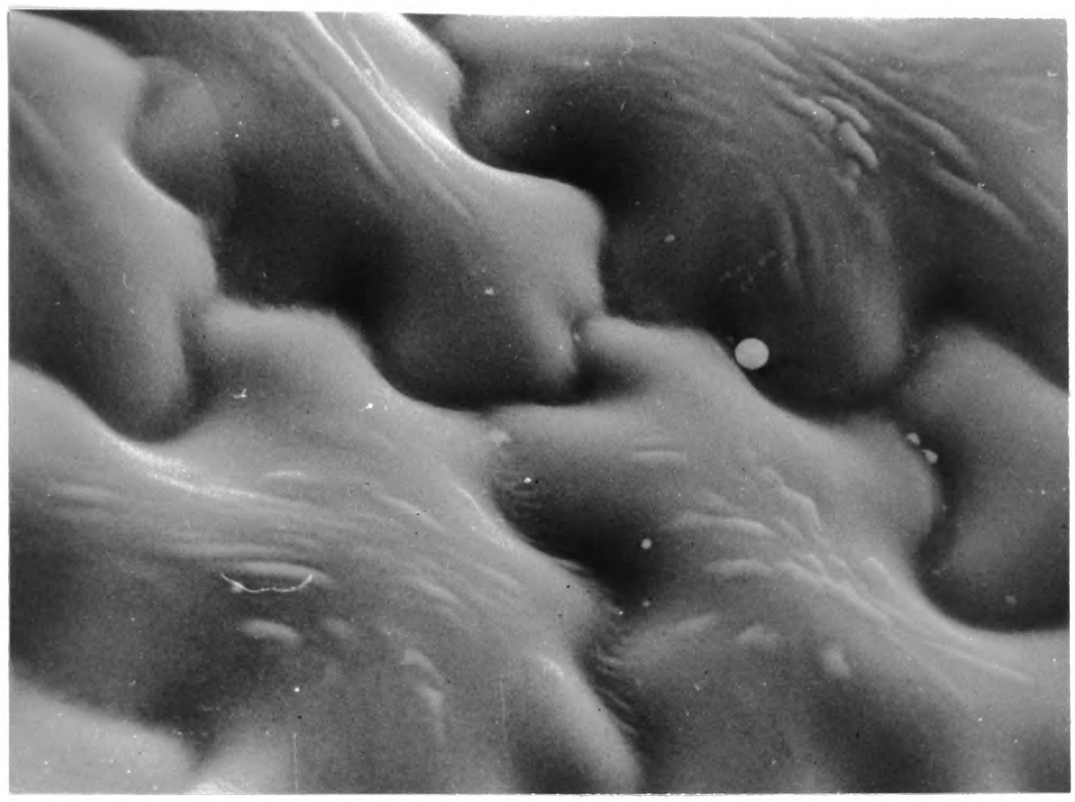


The epidermal cells in the more mature leaves (LPI 1.5) (Plate 8) were considerably larger than those present in the younger leaves (Plates 4, 6). The marked cuticular striations were still evident and would appear to be continuous between the surface of cells on the leaf midrib (Plates 9, 10), but not between cells on the leaf lamina (Plate 11). The cuticle showed a complete absence of crystalline wax deposits. The number of stomata per unit area was very much higher in young leaves, which may be seen by the examination of the abaxial leaf surfaces of A. Nanum at both leaf ages (Plates 5, 12). These plates show 11 stomata on the younger leaves and 5 on the older leaves.

An experiment was set up to investigate if there was any difference in the numbers of stomata per unit area between the mature leaves of the different cultivars, as it had been suggested that this might affect the rust resistant capabilities of the cultivars (Doran, 1921). Leaves were removed from greenhouse grown plants and fixed to microscope slides using Durafix. The leaves were then observed under a light microscope equipped with incident illumination. Both sides of the leaves of each variety were examined, and the numbers of stomata occurring in 10 fields of view (x 650), selected randomly (p.139), were recorded. The mean values of stomatal numbers for leaf surfaces of each species are given (Table 16). A statistical analysis of the data (t-test) showed that there was no significant difference ($p = .05$) between the numbers of stomata present on the equivalent leaf surface of each cultivar.

Plate 8. Scanning electron micrograph of the upper surface of older leaf (LPI 1.5) lamina of A. Nanum (note the absence of surface cuticular wax) (x 580)

Plate 9 Scanning electron micrograph of the upper surface of older leaf (LPI 1.5) midrib of A. Nanum (Note striations appear to continue across cell boundaries) (x 439)



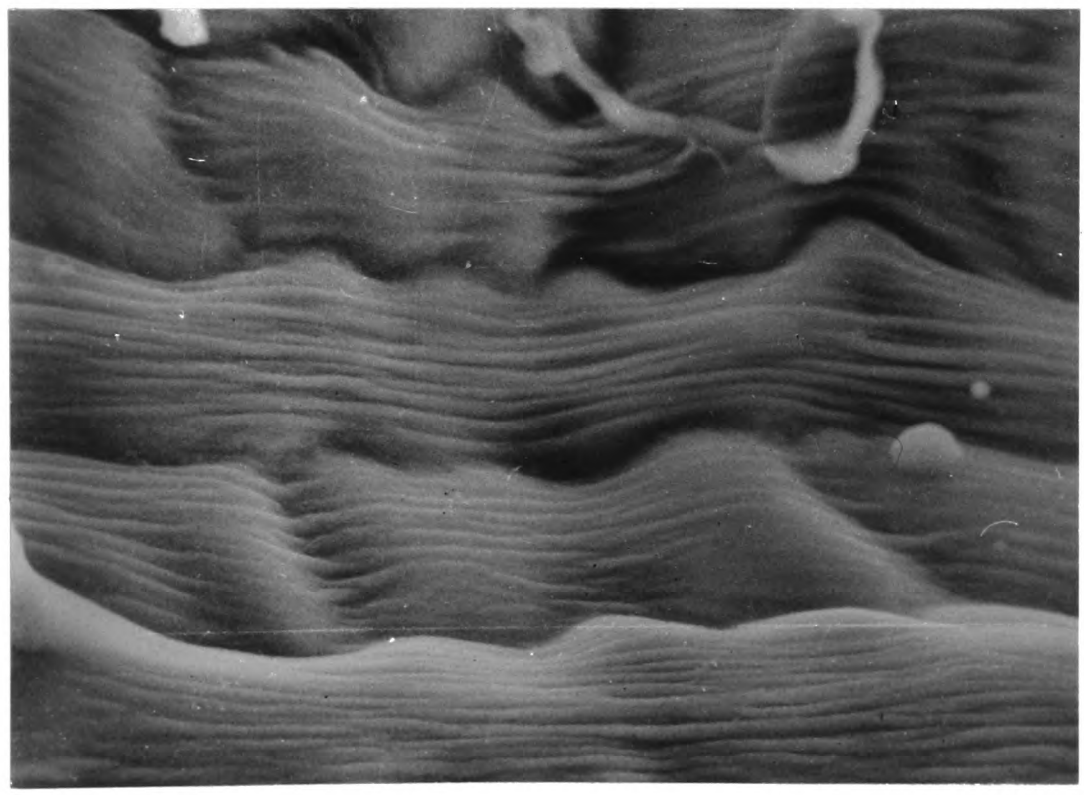


Plate 10 Scanning electron micrograph of the lower surface of older leaf (LPI 1.5) midrib of A. Fi hybrid (note striations appear to continue across cell boundaries) (x 1800)

Plate 11. Scanning electron micrograph of the upper surface of older leaf (LPI 1.5) lamina of A. Fi hybrid showing the absence of surface cuticular wax and the pattern of striation (x 2200)

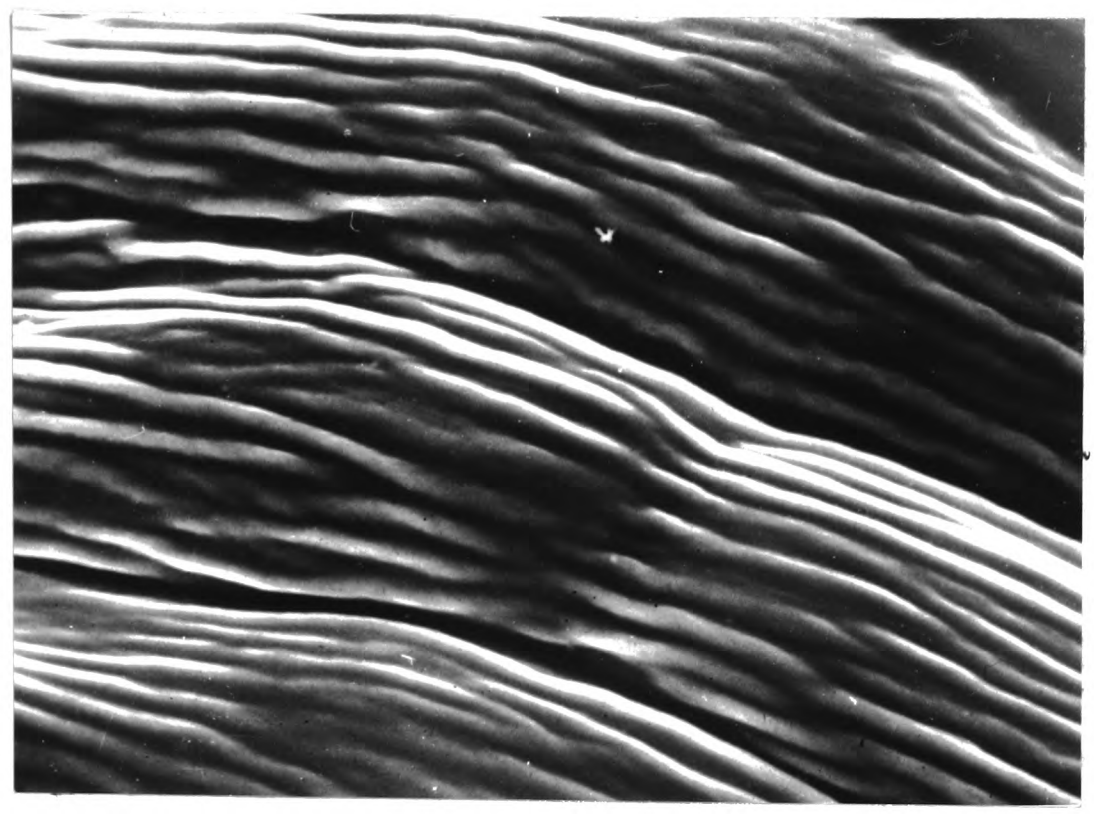


Plate 12 Scanning electron micrograph of the lower surface of older leaf (LPI 1.5) lamina of A. Nanum showing frequency of stomata (x 422)

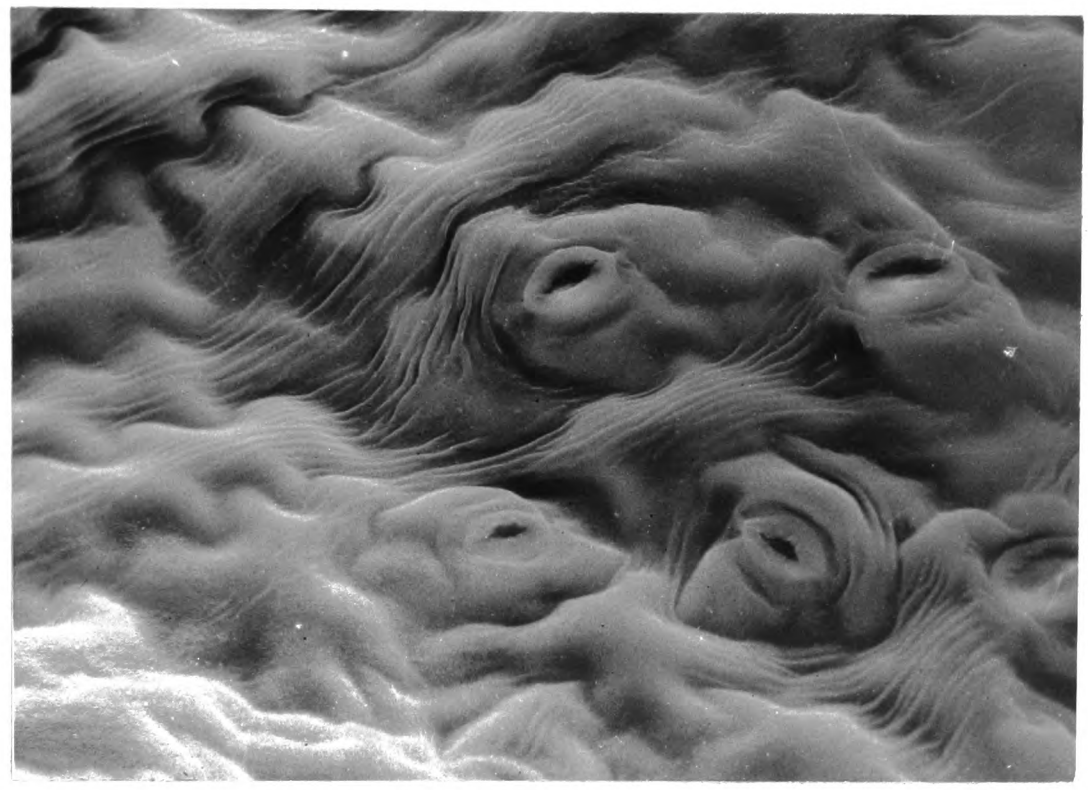


Table 16 Numbers of stomata present on leaves of A. Nanum and A. Fi hybrid

Cultivar	Numbers of stomata per unit area	
	Adaxial surface	Abaxial surface
A. Nanum	3.1 ± 1.1	12.7 ± 0.8
A. Fi hybrid	2.6 ± 0.9	11.5 ± 0.8

EXPERIMENTAL

METHODS

1) DETERMINATION OF A PLASTOCHRON INDEX

The physiologic age of the leaf has been shown to affect the environment on the leaf surface in terms of physical characters (Linskens, 1952) and nutrient status (Schoch, 1955) which may be correlated with the differences in numbers of saprophytic micro-organisms isolated from leaves of different ages (Last, 1955a; Kerling, 1964). Thus leaf age must be taken into account in phylloplane studies. It might appear that the simplest way to observe the effects due to leaf age would be to assume that all the leaves present on a stem at one time constituted an age series and by inference using leaves from different positions would allow observations to be made on the effects of leaf age on the phylloplane microflora. However, the arguments of Richards (1934) mentioned above would seem to preclude the use of such techniques, while his own proposals of using equivalent leaves from different plants might give rise to such variable results that only gross changes would be discernible (Michelini, 1958). The use of a plastochron index (Erickson & Michelini, 1957) would seem to provide a suitable method for the assessment of leaf age which has been used successfully both in studies on plant development (Maksymowych, 1973) and in observations on the phylloplane (Purnell, 1971).

Procedure

Thirty plants of A. Nanum and of A. Fi hybrid were pricked out from Levington compost and planted into 100 mm pots containing U.C. mix D2 compost (Matkin & Chandler, 1957). These plants were grown in a greenhouse under a day length of 16 hours at a constant temperature of 15°C (subject to the variations normally experienced under greenhouse conditions). At the same time each day the lengths of every leaf on all plants were measured to the nearest 0.1 mm using a vernier caliper. These measurements were continued until the flower buds appeared and opened. The data on the leaf length were treated in the following manner. For each plant the mean lengths of the leaves at each node were calculated on a daily basis and the resulting values used to plot graphs of the logarithmic value of the leaf length against time for each node and each plant. The examination of the family of curves for each plant was used to establish the arbitrary reference length necessary for the calculation of the plastochron index of the plant using the formula of Erickson and Michelini (1957).

$$P.I. = n + \frac{\text{Log}n - \text{Log}R}{\text{Log}n - \text{Log}n + 1}$$

where n is the serial number, counting from the base, of the leaf which is just longer than the reference length

R is the reference length

n + 1 is the length of the leaf which is just shorter than the reference length

P.I. Plastochron Index; the age of the plant expressed in plastochrons

The Leaf Plastochron Index (LPI) may be calculated from this.

$$LPI = P.I. - a$$

a is the index number of the leaf under consideration.

2) LEACHING OF SUBSTANCES FROM LEAVES

Many methods have been used to leach substances from leaves. These vary in complexity from the application of single water droplets (Brown, 1922; Deverall, 1967) to spraying the plants with mists of atomised distilled water which, having passed over the plant, are collected, treated and recirculated (Morgan & Tukey, 1964; Purnell, 1971). A technically simpler method in which leaves were detached from the plant and washed in water for a period of time has been used successfully by Blakeman (1968). This method allows more direct techniques of estimating the surface area of dicotyledonous plant leaves from which materials have been leached. The knowledge of the surface area of the leaves which were used enables one to use the relationship proposed by Kovács and Szeöke (1956) for the standardisation of the concentration of leaf washings. A '1 K' (Kovács concentration) solution of leaf washings represents the concentration of water soluble materials that would occur in a film of water 0.1 mm thick over the total surface of the leaf.

(a) Procedure

The procedure adopted was a slight modification of that described by Blakeman (1968). Leaves of a known LPI were removed from test plants and the cut ends of the petioles sealed with molten paraffin wax. These leaves were placed in conical flasks containing 200 ml of distilled deionised water and washed gently on a wrist action shaker (60 rpm) for one hour at room temperature. The resulting solution was filtered immediately through washed membrane filters to remove any micro-organisms present. The leaf washings were then evaporated to dryness in a rotary evaporator at

35°C. In the meantime the surface area of the leaves had been calculated on a leaf area machine (Hayashi Denco Manufacturing Co.). The total surface area was used to calculate the volume of deionised water to be added to give a solution of 1 K concentration (Kovács & Szeöke, 1956).

(b) Analysis of Leachates

Analysis of the leaf leachates was restricted to the estimation of the quantities and identification of the carbohydrates and amino acids present. These substances account for major quantities of leached material (Tukey, 1970) and these might be expected to act as a source of nutrient for micro-organisms living in the phylloplane (Blakeman, 1971).

Solution of 1 K concentration were used for the quantitative analysis of the leaf washings. The separation of the leachates into amino acid and sugars for the purposes of analysis is unnecessary since McBride (1970) found that no interference to the analytical techniques occurred between these groups of compounds.

i) Quantitative analysis

Carbohydrates: The carbohydrate content of leachates was assessed by the Anthrone colour reaction (Yemm & Willis, 1954) applying the modifications of Deriaz (1961). This test is specific for sugars. The carbohydrate standards consisted of a series of solutions containing equal quantities of sucrose, glucose and fructose. This mixture was used to simulate the leaf sugar mixture expected in leachates (Shiroya et al., 1962; Purnell, 1971). The standard series contained 0.0001 to 0.1 g of each sugar or 0.0003 to 0.3 g of total carbohydrate per litre. These solutions were used to

calibrate the spectrometer (Evans Electroselenium) at 625 μm so that the intensity of the anthrone colour produced by leachate solutions could be directly related to the amount of carbohydrate present.

Amino acids: The total concentration of amino acids in leachates was analysed using the ninhydrin colorometric analysis described by Rosen (1957). Ninhydrin reacts quantitatively with several classes of compounds including amino acids, imino acids, amino alcohols and primary amides.

The colour intensity attained with amino acids varies only over a range of 5% between Lysine and Tyrosine (Rosen, 1957). The standard series used to relate colour intensity with amino acid concentration consisted of equal proportions ($g\ l^{-1}$) of alanine, aspartic acid and glutamic acid since these are the amino acids found in greatest abundance in the leachates of many plants (Nykvist, 1963; Morgan & Tukey, 1964; Deverall, 1967). These amino acids give a stable purple colour with ninhydrin which is read at 570 μm in a spectrometer. One complication was that one set of test samples consistently produced a yellow colouration with ninhydrin owing to the presence of proline or hydroxyproline (Rosen, 1957). This necessitated setting up a second standard series containing equal quantities of proline and hydroxyproline. The resultant colouration with ninhydrin was read at 440 μm in the spectrometer (Rosen, 1957).

ii) Qualitative analysis

Qualitative analysis of the leaf leachates was done using thin layer chromatography (Randerath, 1968).

Carbohydrates: Thin layer chromatography plates were made using Kieselguhr G (Merck Chemicals) buffered with 0.1 N boric acid, (30 g Kieselguhr G plus 60 ml 0.1 N boric acid) and activated by heating at 108°C for 1 hour. Two dimensional chromatography was used to separate the carbohydrates. The solvents used were:-

First direction: n-Butanol:Acetone:Water (4:5:1)

Second direction: Methyl ethyl ketone:Acetic Acid:Water (3:1:1)

The chromatograms were developed by spraying with a freshly prepared anisaldehyde sulphuric acid mixture (0.5 g anisaldehyde; 5 ml glacial acetic acid; 0.1 ml concentrated sulphuric acid) heated to 108°C for 5 to 10 minutes. The standards used for this test comprised water solutions of known sugars applied at a rate of 5 µg per spot.

Amino acids: Two dimensional chromatography on unbuffered layers of Kieselguhr G layers was used for the separation and identification of amino acids. The standards comprised water solutions of known amino acids applied at a rate of 5 µg per spot. The solvents used were:-

First direction: Chloroform:Methanol:17% Ammonium hydroxide (2:2:1:)

Second direction: Phenol:Water (75:25 w/w) (20 mg sodium cyanide per 100 g mixture)

Chromatograms were developed by spraying with a ninhydrin acetic acid mixture (0.03 g Ninhydrin; 10 ml n-Butanol; 0.3 g acetic acid) and heating the chromatogram to 110°C for 10 minutes. The colours and positions of the spots were noted immediately as they tend to fade rapidly.

RESULTS

1) LEAF DEVELOPMENT AND THE FORMATION OF A PLASTOCHRON INDEX

The daily measurements of leaf length for both A. Fi hybrid (Fig. 18) and A. Nanum (Fig. 19) are given in graphical form as the logarithmic value of leaf length plotted against time. Each figure represents the results for one plant given as an example. The families of curves plotted for the other plants of the same cultivar were very similar. Each figure shows a family of curves which are very nearly straight and parallel over a considerable time interval and are fairly equally spaced along the horizontal time axis. The slope of the curves and the final leaf length for A. Fi hybrid were greater than those found for A. Nanum. From these figures a reference length of 15 mm was chosen for use in the plastochron index, as at this length the leaves were growing exponentially and the curves were nearly parallel.

2) ANALYSIS OF LEAF LEACHATES

Results of the quantitative analysis of the leachates for carbohydrates (Table 17) and amino acids (Table 18) showed that larger amounts of these substances were present in the leachates from the older leaves. Also, the leachates from the older leaves of A. Nanum (LPI 1.5) contained about 11 times the quantity of carbohydrate as the leachate from comparable leaves of A. Fi hybrid. However the latter contained relatively greater amounts of amino acids.

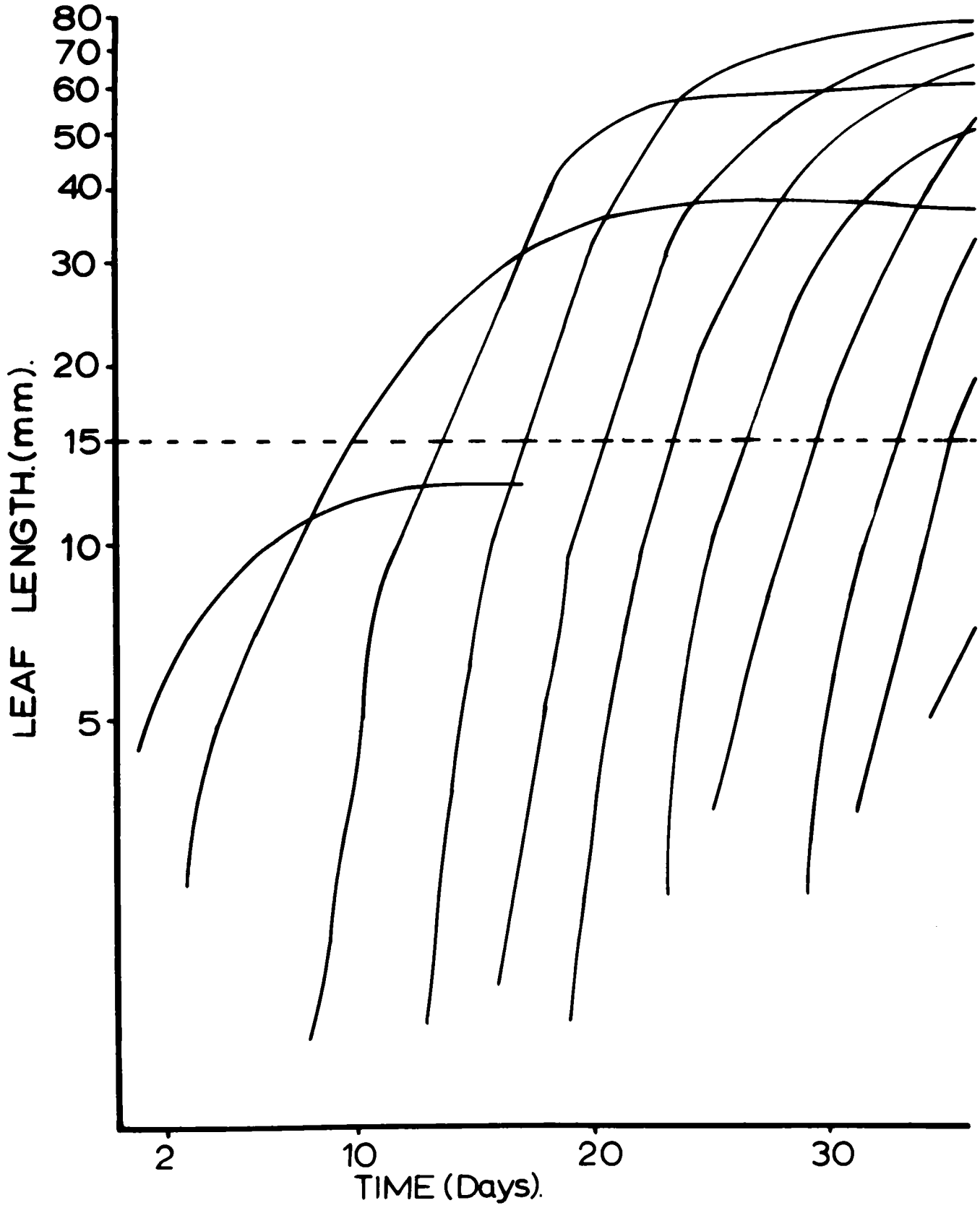


Fig. 18 A. Fi hybrid: Increase in length of successive pairs of leaves with time

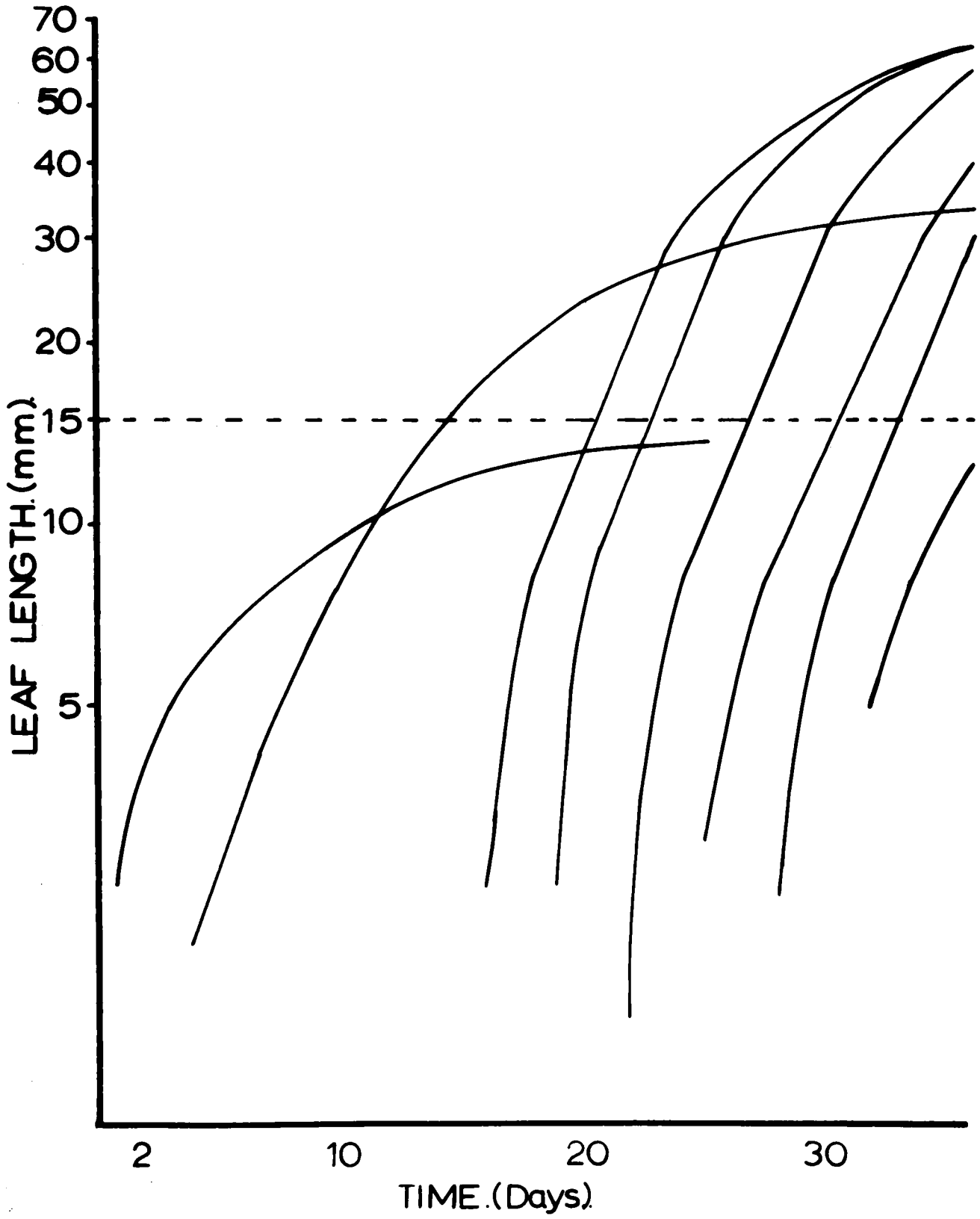


Fig. 19 A. Nanum: Increase in length of successive pairs of leaves with time

Qualitative analysis of the leachates revealed that the same substances were in the leachates from young and old leaves for each cultivar. All four groups of leachates contained the sugars fructose, sucrose and glucose. However, there was a difference in the amino acid content of leaves of each cultivar of antirrhinum (Table 19), in that the leachates of A. Fi hybrid contained proline and hydroxyproline in addition to the amino acids found in the leachates from A. Nanum. The effects of these leachates on some saprophytic micro-organisms and P. antirrhini, especially the inhibitory activity of the A. Fi hybrid leachate on the last organism will be considered later (p. 152)

Table 17 Quantity of total carbohydrates present in leaf washings from A. Nanum and A. Fi hybrid

	Carbohydrate concentration mg l^{-1}	
Leaf age (LPI)	- 0.5	1.5
A. Nanum	0.08	7.3
A. Fi hybrid	0.02	0.68

Table 18 Quantity of total amino acids present in leaf washings from A. Nanum and A. Fi hybrid

	Amino acid concentration µg l^{-1}	
Leaf age (LPI)	- 0.5	1.5
A. Nanum	0.24	0.63
A. Fi hybrid	0.38	1.43

Table 19 Types of amino acid present in leaf washings of A. Nanum and A. Fi hybrid

Amino acid	Cultivar	
	A. Nanum	A. Fi hybrid
Alanine	+	+
Aspartic acid	+	+
Glutamic acid	+	+
Serine	+	+
Proline	-	+
Hydroxyproline	-	+

ASEPTIC CULTURE OF PLANTS

The growing of plants under aseptic conditions, for brief periods of time, is not new. Several methods have been tried in the past, but in most cases only small plants or parts of plants have been involved. A number of workers have grown individual plants in glass cylinders (German & Bowen, 1951; Kathrein, 1951; Szember, 1959) whilst others have used a container in which only the roots were under sterile conditions (Blanchard & Diller, 1950). Partial aseptic conditions were attained by Leben (1961) when growing cucumber plants in plastic film isolators. This technique has been refined considerably by later workers investigating the effects of soil fungi on the growth of several different plants (Lindsey, 1967; Lindsey & Baker, 1967). The use of film isolators has not proved so effective in studies on the aerial parts of plants as the process of inoculation of the plant at later stages of growth has led to a high incidence of contamination of the plants by unwanted micro-organisms, owing to the necessity of opening large areas of the isolator to allow access to the plants (Lindsey, 1970). One apparatus having removable panels and glove ports was described by Estey and Smith (1962), but this apparatus used two large chambers which, although they could contain many plants, would be very susceptible to cross contamination between plants, especially where experiments use fungi which may produce spores which could be dispersed through the air. A scheme for growing single plants in wide necked Erlenmyer flasks was devised by Marx and Zak (1965) for use in studies on mycorrhizal development on roots. This method was used by McBride (1970) to study the colonisation of larch leaves by

phylloplane micro-organisms.

All of the methods for growing aseptic plants described here rely on the surface sterilisation of seed to ensure that the seedling itself is free from contamination. Chemical sterilisation is used most commonly. Agents such as hydrogen peroxide (Trappe, 1961), peracetic acid (Lindsey, 1967), and sodium hypochlorite (C.M.I., 1968) have been used. All three of these chemicals were tried in the sterilisation of the surface of antirrhinum seeds. Hydrogen peroxide was ineffective at concentrations below 30% (15 minutes immersion). If the concentration was raised or the immersion time extended the seeds were killed. The peracetic acid gave indifferent results but the use of hypochlorite solution (1% available chlorine) for 10 minutes proved effective in the majority of cases. After these treatments the seeds were washed three times in sterile distilled water and incubated on agar media at 18°C to test for contamination. Although Nutrient agar (Oxoid) was effective in the detection of contaminants, the antirrhinum seeds would not germinate on this medium. However, seed germination and contamination testing were satisfactory using Czapek Dox agar (Oxoid 0.5 strength medium) or tap water agar. The germinated seeds were examined using a binocular microscope and uncontaminated seeds transferred to a growth medium.

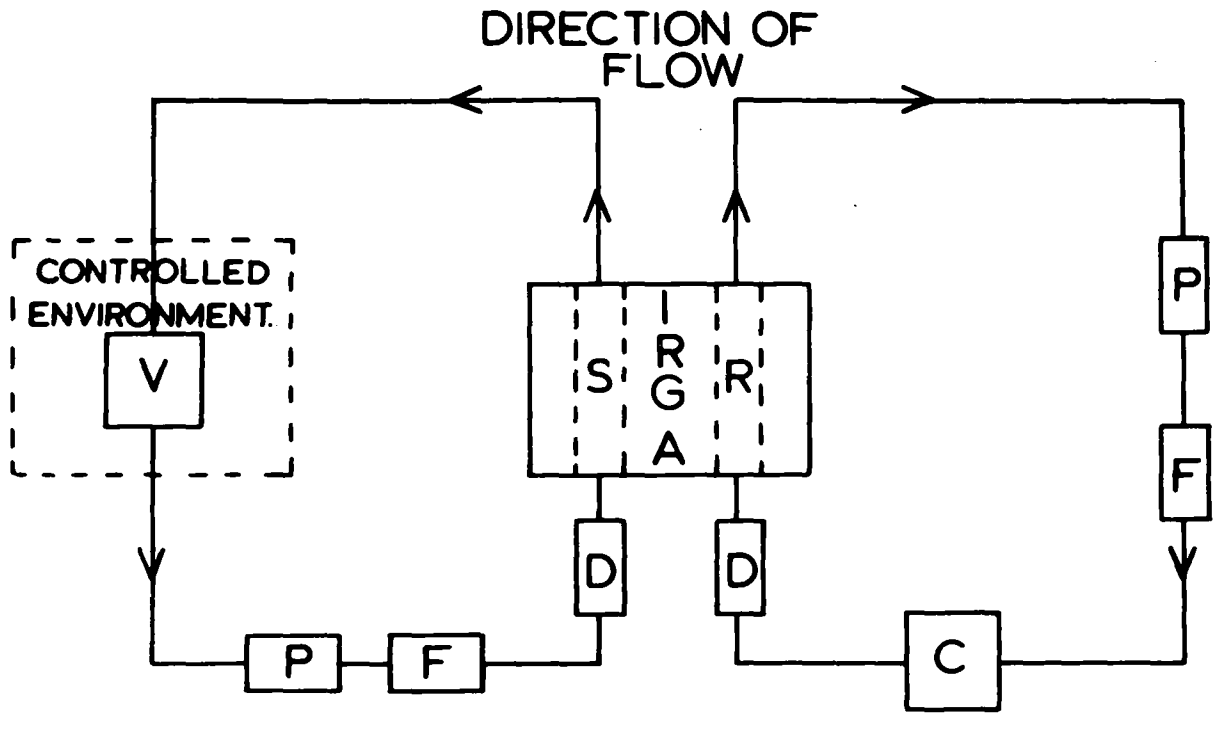
Growth media which have been used previously in aseptic plant culture include garden soil (Estey & Smith, 1962), quartz sand or perlite sand mixture (Lindsey, 1967), and peat vermiculite mixtures (Marx & Zak, 1965; McBride, 1970). Trials were conducted using the above growth media and also a peat sand compost (UC mix D2, Matkin & Chandler, 1957), which had been shown to support good growth of

antirrhinums (p. 83). Samples of these media were autoclaved at 121°C for 45 minutes (McBride, 1970) or at 121°C for 8 hours (autoclaved for 4 hours, cooled for 24 hours, then reautoclaved for 4 hours) (Lindsey, 1967), and then placed in pots in a greenhouse. The plants placed in the samples of the above media showed very poor growth. An alternative method of sterilisation was tried in which dried samples of the growth media were treated by γ irradiation at a rate of 5 Mrads. Antirrhinum plants grew well in all soil samples treated in this way. The use of composts was adopted in preference to garden soil as a uniform growth medium would be required for different experiments initiated over a considerable period of time. As both vermiculite and perlite were difficult to obtain at this particular time the peat sand compost was used in the subsequent tests.

Several types of enclosures have been used for the culture of aseptic plants. The idea of using large containers was rejected as some of the proposed experiments involved the use of the fungi P. antirrhini and C. cladosporioides whose spores are dispersed readily through the air under natural conditions (Walker, 1955; Pady et al., 1969). Thus it was highly probably that cross contamination could occur between plants within an enclosure. McBride (1970) had grown single plants in Erlenmyer flasks stoppered with cottonwool bungs. This simple method was tried initially. Dry irradiation sterilised compost (250 mls) was placed in each sterile 21 wide necked conical flask and 200 mls of sterile deionised water added. A germinated microbe-free seedling was transferred to the flask using sterile forceps. The flasks were stoppered with sterile cottonwool bungs and placed in an environment room set to give 18

hour 18°C days and 18°C nights. Many seedlings failed to take in the flask culture, but those that did take only grew slowly to the stage at which two pairs of true leaves were produced and then growth ceased. Careful manipulation of the temperature, water, nutrient regimes and daylength failed to reinitiate growth. The cottonwool bungs in some of the flasks were replaced with very thin polythene film to allow more light into the flasks, but still no further growth was observed.

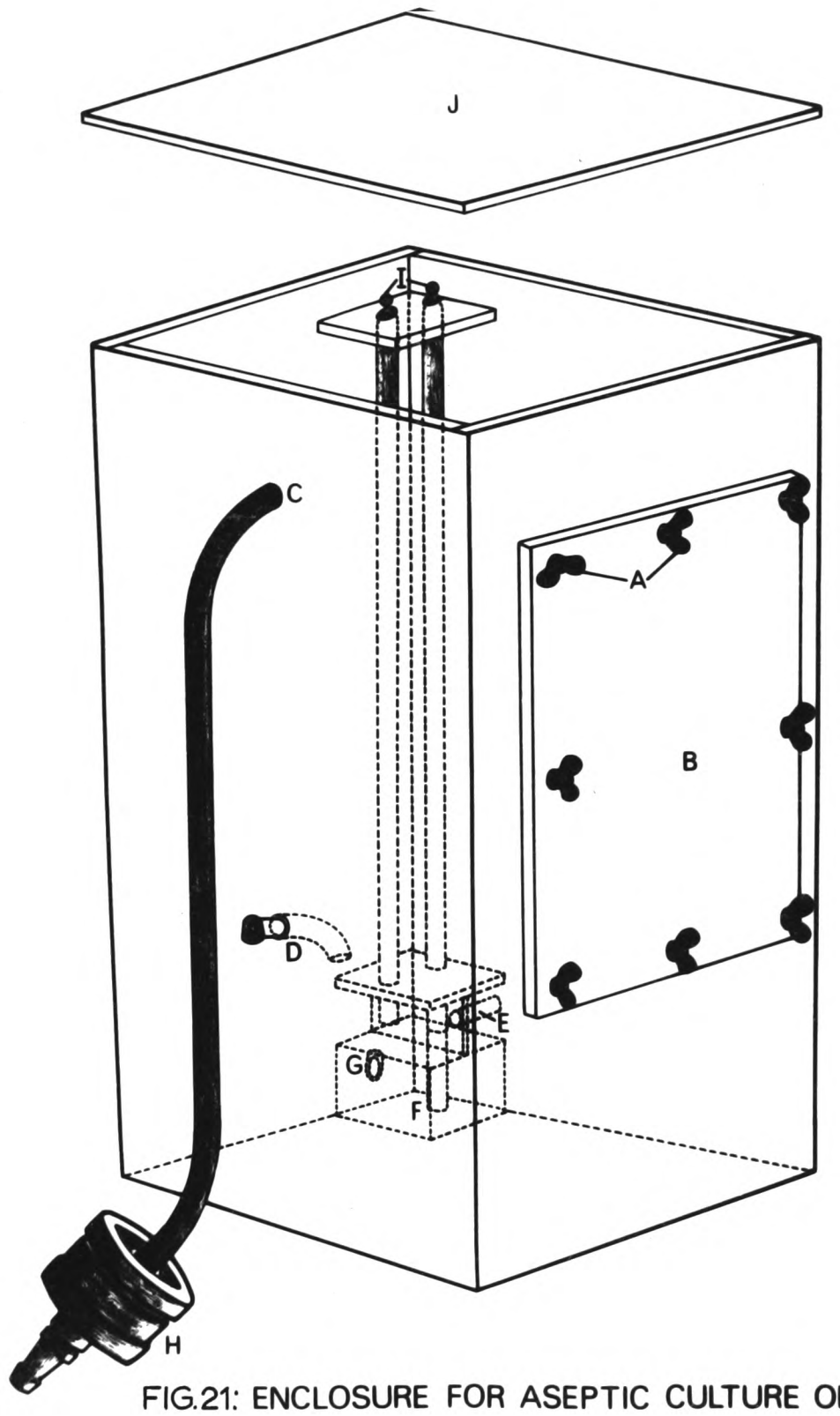
Accordingly, an experiment was set up to investigate the atmospheric conditions within the flasks, in particular the concentration of carbon dioxide. The seedlings were placed in compost contained in 2 L wide necked side arm flasks. The top was plugged with cotton wool and the side arm connected to two concentric tubes which were linked to an Infra Red Gas Analyser (Grubb Parsons). This apparatus was used to monitor the carbon dioxide concentration within the flask (Fig. 20 diagram of apparatus). It was found that the carbon dioxide concentration dropped from 300 ppm to 30 ppm within a period of 25 minutes after the lighting in the environment room had been switched on. At these low levels probably only intercellular carbon dioxide would be present (Grace, personal communication), which would inhibit photosynthesis and prevent normal growth. The introduction of filtered air to the flasks did overcome this problem of carbon dioxide deficit and allow the plants to grow. However, the use of many conical flasks which allowed only very restricted access to the plants both for inoculating the leaves and their recovery for examination was not really suitable for continuous experimentation.



I.R.G.A. Infra Red Gas Analyser.
S Sample Column.
R Reference Column.
D Drying Tower.
F Flowmeter.
P Pump.
C CO_2 Scrubbing Column.
V Flask containing Plant.

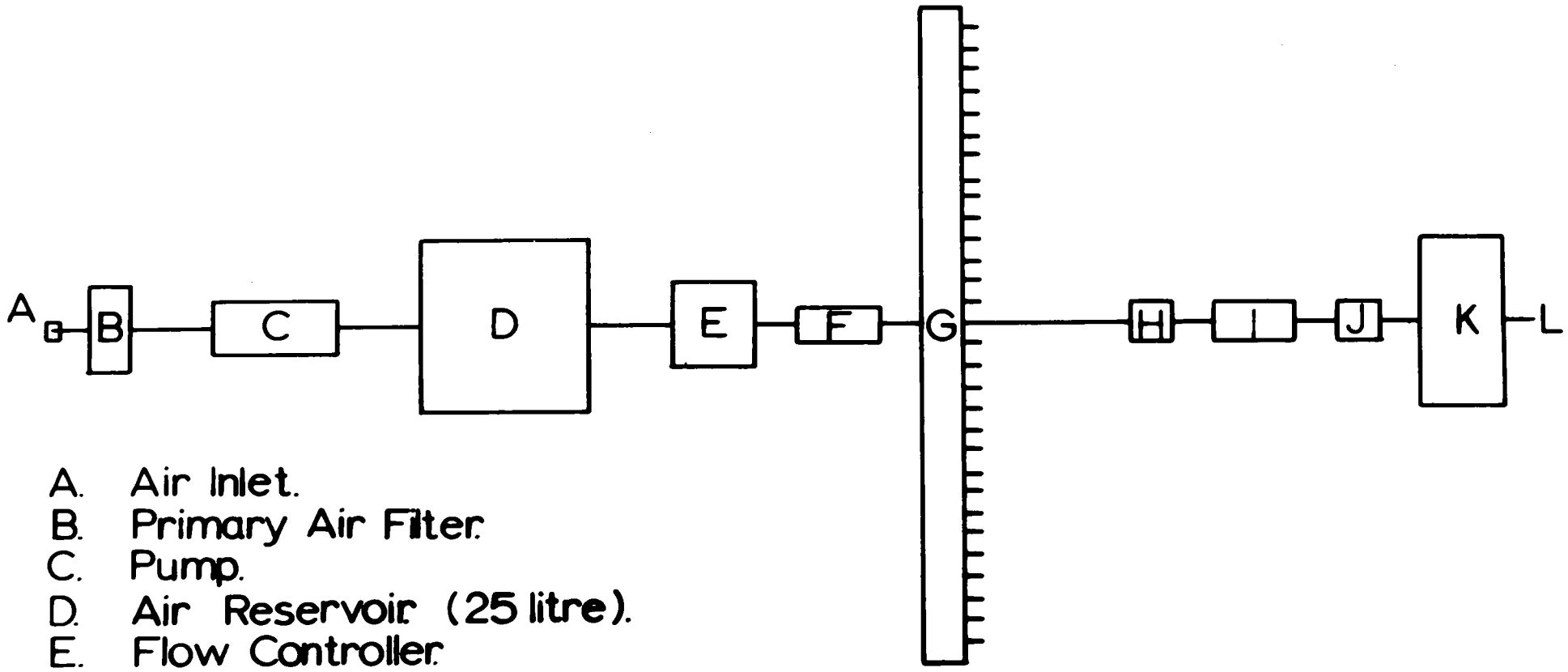
Fig. 20 Flow diagram of apparatus for measuring carbon dioxide levels in the atmosphere surrounding plants in flask culture

An enclosure for the culture of single plants was designed (Fig. 21). This allowed easy access to the plant leaves at several nodes. The box was constructed from clear 6 mm perspex (ICI Limited) and provision was made for a supply of filtered air and the monitoring of temperature and humidity conditions within the container. Air was supplied to the box at a rate of 1 l min^{-1} via a membrane filter (0.45 µm pore size Gelman Hawksley Limited). The air passed through the box and exited via a glass-fibre filter. The exit was so positioned that the stream of air leaving the box aspirated the bulbs of wet and dry bulb thermometers. These thermometers were specially made short stem (250 mm) short range (0-25°C) instruments marked with a 0.1°C scale (McKenzie & Sons, 54 Northbury Road, Thornton Heath, Surrey). This fine scale enabled the wet bulb temperature depression to be determined with some accuracy. All normally available thermometers reading to 0.1°C had stem lengths in excess of 450 mm which would have meant building more expensive boxes of a size which was larger than that required, and than would fit easily into the controlled environment space available. In order to obtain a sufficiently large number of leaves of the same age for experiments a minimum of 30 plants was considered necessary and so 30 chambers of the type described above were constructed. The air supply for these boxes was supplied by a single pump (Charles Austin Duplex 2) which fed a flow controller and meter (G.A. Platon). The metered supply was passed through a manifold from which 30 separate supplies could be drawn. The flow rate in these 30 channels was monitored and balanced by short range flow meters equipped with needle valves (G.A. Platon) to ensure that the same flow rate was applied to each box (Fig. 22). It was hoped to instal humidity control into this



A Wing nuts to secure panel
B Removeable panel
C Air inlet pipe
D Water inlet pipe
E Air outlet
F Reservoir for wet bulb thermometer
G Subaseal
H Air filter
I Wet & Dry thermometers
J Removeable top

FIG.21: ENCLOSURE FOR ASEPTIC CULTURE OF SINGLE PLANTS



A. Air Inlet.
B. Primary Air Filter.
C. Pump.
D. Air Reservoir (25 litre).
E. Flow Controller.
F. Flow Meter.
G. Manifold Outlets. (30).
H. Needle Valve.
I. Flow Meter.
J. Membrane Filter.
K. Perspex Box.
L. Air Outlet.

Fig. 22 Flow diagram: Air supply for multiple aseptic plant culture

system, but none of designs tried up to the present time gave adequate control.

PROCEDURE USED FOR SETTING UP PLANTS FOR ASEPTIC CULTURE

Great care was taken at all stages to try to reduce the risks of contamination to a minimum. To this end all activities involving the opening of a box were done on a laminar flow bench (Pathfinder, Havant, Hants.). The inside of the box was swabbed with acidified hypochlorite solution. A 250 ml pyrex beaker, sterilised by autoclaving, was filled with 200 ml of the sterile compost. The outside of the beaker was swabbed with hypochlorite and then placed in the box the top of which was sealed using transparent scotch tape. The air filter and PVC connecting tube (sterilised by autoclaving) were connected to the box. Likewise the sterile tubing and glass filter were connected to the outlet of the box. The box was left in this condition for 24 hours before being connected to an air supply. Filtered air was passed through the box for 24 hours to remove any chlorine remaining. Then 100 ml of sterile deionised water was added to the compost via subaseal D (Fig. 21). The reservoir for the wet bulb thermometer was also filled with sterile water via subaseal G. After a further 24 hour period the front panel of the box was removed and a clip applied to the air exit tube so that the box was under slight positive pressure. An aseptic seedling produced by the methods described above was placed in the compost using sterile forceps and the box resealed and placed in a controlled environment room.

By these means aseptic plants could be produced. However, several problems arose which precluded the setting up of any full

scale experiments using this system. The germination percentage of antirrhinum seeds was generally low, but during the period when these experiments were being attempted, germination fell to about 5%. Initially this was thought to be due to the surface sterilisation procedures, but this did not prove to be the case as untreated seeds showed an equally low germination percentage. Fresh seeds, which gave a higher germination rate, were obtained after a considerable delay. However, the supply of sterile seedlings was always low owing to both the occasional failure of the surface sterilisation procedures and to the failure of some plants to establish themselves in the compost within the boxes. The established plants within the boxes were very susceptible to contamination which seemed to occur after the plants had reached the height of 100 mm. This happened to about six plants in 30 on several occasions. The source of this contamination has not yet been traced.

Not all the problems experienced were due to difficulties with the plants. Failure to maintain the humidity at levels in excess of 80% R.H. even within environmental rooms provided with some form of humidity control meant that the environment at the leaf surface might not have been amenable to the establishment of saprophytic micro-organisms as had been observed both in dry field and greenhouse conditions (Leben & Daft, 1967).

These difficulties meant that insufficient plants bearing leaves of similar plastochron age were available at any one time to enable a statistically meaningful experiment to be set up. Thus, this section is devoted to the description of the development of a system for growing antirrhinum plants under aseptic conditions. It

is hoped that a refined version of this system will enable full scale experiments, on the colonisation of leaves, and the interaction of micro-organisms on the leaf surface to be set up in the future.

DISCUSSION

The examination of the data on leaf growth (Figs. 16, 19) led to the adoption of a reference length of 15 mm for use in determining the age of leaves and plants using a plastochron index (Erickson & Michelini, 1957). At this point the curves showed that the leaves were growing exponentially, the curve for each leaf pair was very nearly parallel, and the leaves were large enough to measure with some accuracy (0.1 mm) without causing injury to the leaf or shoot. Also leaves both shorter and longer than 15 mm were always present on some sequential pair of nodes which is necessary for the calculation of the plastochron index of the plant at any point in time (Erickson & Michelini, 1957). These curves and the calculated values of the plastochron index revealed that there was a difference between the cultivars of antirrhinum in terms of rate of development of both plants and leaves. A. Fi hybrid plants had a plastochron index of 7.17 after 30 days whereas the A. Nanum plants had a plastochron index of only 5.06 after the same time period. Also the first pair of leaves reached the reference length in 10 days in A. Fi hybrid but took 14.5 days in A. Nanum. For later leaves the time interval between successive pairs of leaves reaching the reference length was a regular 3 days in A. Fi hybrid, but varied from 2.6 to 4 days in A. Nanum. Also leaf growth was faster in the hybrid as at the end of the exponential growth phase the LPI was 1.54 whereas in A. Nanum the LPI was 1.79. Thus the leaves in the hybrid were 0.25 plastochrons younger at this growth stage.

Although differences between the cultivars in terms of plant and leaf development rate were observed the physical characters of

the leaves of both cultivars were similar. Some workers have demonstrated that the exponential growth of leaves is due to cell enlargement (Richards, 1969; Maksymowych, 1973) as cell division practically ceases as the buds open (Gregory, 1956). There seems no reason to suppose that leaf growth in antirrhinums would be any different and a possible indication of this phenomenon may be seen in the increase in size of epidermal cells as the leaf ages from LPI - 0.5 to 1.5 (Plates 4, 8). Accompanying the increase in cell size is a corresponding decrease in the numbers of stomata per unit area of leaf (Plates 5, 12), which would be the expected result of the expansion of all cells in the lower epidermis. In plants of both cultivars, the numbers of stomata per unit area were higher on the abaxial surface, confirming the observations on many other plant species (Meidner & Mansfield, 1958). Although the mean values of stomata per unit area were slightly different for the two cultivars (Table 16), the variation around these mean values was so great that this difference was not significant ($p = .05$).

The finer surface characteristics of both cultivars of antirrhinum were similar. Both the upper and lower surfaces of the leaves showed marked cuticular striations (Plates 4, 5) which appear to be continuous between the cells in the midrib area (Plate 9) but not on the leaf lamina (Plate 11). Similar patterns of striation have been observed on the lower leaf surface of Trifolium repens and the upper leaf surface of Acer pseudoplatanus L and Syringa vulgaris (Holloway, 1971). Crystalline surface wax was absent from both leaf surfaces (Plates 5, 8) and so the leaves should not be markedly hydrophobic (Juniper, 1959) and may be susceptible to leaching (Cholodny, 1932). The very low cuticular

resistance of Antirrhinum spp. to the passage of water vapour found by Milthorpe (1961) may be due to this lack of surface wax.

The surface of the leaf was found to be very wettable as a drop of water placed on the leaf would spread out immediately, which should make it susceptible to leaching (Fogg, 1947). Carbohydrates and amino acids were found in the leaf washings from both cultivars but the amounts obtained from young leaves (LPI - 0.5) were less than those obtained from the more mature leaves (LPI 1.5) (Table 17). This difference was more pronounced in the case of carbohydrates. Also, the two cultivars showed a considerable difference in the levels of carbohydrates leached from the mature leaves, A. Nanum losing about 11 times the quantity that was lost from A. Fi hybrid. It is more or less impossible to obtain a direct comparison between the quantities of leached material observed here and those values obtained from different plants by other workers owing to the multiplicity of the units which have been used. Some examples of the quantities of carbohydrates obtained from other plants are: 800 Kg Hectare^{-1} year^{-1} from apple trees (Dalbro, 1956), 6% of the dry weight equivalent of young bean leaves (Tukey et al., 1958), 5 µg ml^{-1} from bean seed cavities (Deverall, 1967), 2 µg g^{-1} dry weight from larch (McBride, 1970), and 0.3 µg cm^{-2} from mature swede leaves (Purnell & Preece, 1971). Clearly there is a good case for the standardisation of units used to express the quantities of substances leached from plants. Here an attempt has been made to relate the concentration of leaf washings to the levels which may occur on the leaf surface, by using the method of Kovacs and Szeőke (1957), and also to assign a physiologic age to the leaf using the leaf plastochron index

(Erickson & Michelini, 1957). The susceptibility of antirrhinum leaves to leaching increased with age. This situation has been found in other plants including apple, barley, carnations, sugar-beet, lime and beans (Arens, 1934; Lausberg, 1955; Stenlid, 1958).

The qualitative analysis of leachates showed that glucose, fructose and sucrose were leached from antirrhinum leaves. These sugars have been found as the predominant carbohydrate component of leachates of several plants (Shiroya et al., 1962; Deverall, 1967; McBride, 1970; Purnell, 1971). Similarly the amino acids obtained in the leachates in this study (Table 19) have been obtained from many plants (Morgan & Tukey, 1964). However, the amino acid content of the leachates of the two cultivars of antirrhinum was different. Only A. Fi hybrid leaf washings contained proline and hydroxyproline. These were present as the largest amino acid component. This could be judged by the yellow colouration of the ninhydrin reagent (p.103). This was caused by the presence of these two amino acids (Yemm & Willis, 1954). The leachate solution gave maximum absorption at 440 µm and only a very small peak was observed at 550 µm, which is the maximum absorption band for the ninhydrin reaction with all other amino acids. This was confirmed using a spectrometer (Pye Unicam SP900). The only other record of hydroxyproline occurring in plant leachates is in those from carnations (Morgan & Tukey, 1964).

Whilst the rate of leaf development and leaf leachates differed between these two cultivars of antirrhinum, the leaf surface characteristics were very similar. Although Doran (1921) suggested that the susceptibilities of cultivars of antirrhinum to P. antirrhini were related to the higher numbers of stomata on the adaxial surface of the leaf relative to the resistant cultivars, the observations

presented here would not support this hypothesis. However, the relationship of cultivar susceptibility to fungal disease in castor beans has been correlated to the higher quantities of carbohydrates leached from the leaves (Orellano & Thomas, 1962). This might have a bearing on the rust susceptibility of the cultivars of antirrhinum as the leaf washings of the susceptible A. Nanum have been shown to contain larger quantities of carbohydrates than the rust resistant cultivar. It should be noted that the observations on castor bean plants were in relation to a facultative pathogen rather than an obligate rust pathogen the germination of which has been shown to be unaffected by external sources of carbohydrate in vitro (p. 72). Later work showed that the leachate from the rust resistant cultivar inhibited P. antirrhini markedly (p. 153).

SECTION 3 PLANT-MICROBE INTERACTIONS

SECTION 3

PLANT-MICROBE INTERACTIONS

INTRODUCTION

The activity of micro-organisms on leaf surfaces has been much discussed, but no clear cut conclusions have been reached, particularly concerning the activity of filamentous fungi on green leaves (Kerling, 1958; Last & Deighton, 1965; Dickinson, 1965, 1967; Fokkema, 1968; Lamb & Brown, 1970; Ruscoe, 1971; di Menna, 1971; Pugh & Buckley, 1971a; Bainbridge & Dickinson, 1972). The commonest inhabitants of the phylloplane are the budding Sporobolomyces roseus and filamentous Cladosporium spp. (Last & Deighton, 1965; Last & Warren, 1972). The occurrence of these organisms has been related to plant age and more usually to season. The effect of season has been explained in terms of the abundance of spores of these organisms occurring in the atmosphere at different times of the year (Last, 1955a; Gregory & Hirst, 1957; Hamilton, 1959; Kerling, 1964; Holloman, 1967; Harvey, 1967). However, there is some evidence for the activity of S. roseus and Cladosporium spp. on green leaves. The increasing numbers of S. roseus occurring on ageing leaves were found to be similar in summer and in winter months when few spores were present in the atmosphere, (Last, 1955a). Actively sporulating colonies of Cladosporium spp. have been observed on plants in the field (Pugh & Buckley, 1971a; Diem, 1974).

Several workers have demonstrated that saprophytic micro-organisms affect the activities of fungi and bacteria attempting to infect aerial parts of plants (see Leben, 1965a; Sihna, 1965).

More recent work has suggested that interactions may occur which cannot simply be attributed to direct microbial antagonism. Among these are the effects of pollen on antagonism (Warren, 1972a; Fokkema, 1973) and the response of the plant itself to the presence of non-pathogenic micro-organisms (van den Heuvel, 1969).

In the work described in this thesis, a combination of *in vitro* and *in vivo* techniques were used to study in detail the initial colonisation of green leaves, of defined physiologic age, by saprophytic microbes, and also the interactions which might occur between saprophytes and pathogens during the early stages of plant infection.

REVIEW OF LITERATURE

INTERACTIONS AND ACTIVITY OF MICRO-ORGANISMS ON THE LEAF SURFACE

Much work has been done on the antagonistic interactions which have been shown to exist between some pathogenic and saprophytic micro-organism. The bulk of this research has been concerned with root or soil inhabiting micro-organisms, but more recently there are increasing numbers of reports of interplay between saprophytic phylloplane micro-organisms. This evidence suggests that such antagonism exists not only between the major groupings of micro-organisms but also within them. Populations of S. roseus, having been isolated previously from the entire surface of wheat leaves, were suddenly found to be restricted to the leaf margin while the white yeasts Tilletiopsis spp. also members of the Sporobolomycetaceae, were isolated from the remainder of the leaf lamina (Last, 1955a).

Another example of possible antagonism between saprophytes was observed during the colonisation of sycamore leaves by A. pullulans. This organism was found to grow actively until the numbers of Cladosporium spp. and Epicoccum nigrum began to increase. At this point in time the form of A. pullulans changed from its actively growing form to a pigmented and thick walled resting structure, which readily germinated on culture media (Pugh & Buckley, 1971a).

An important purpose of the present investigations was to find antagonists of plant pathogens which could be used in the control of plant disease. Although antagonistic micro-organisms have been found, their use in disease control has not proved productive owing to their inability to withstand adverse field conditions (Leben et al., 1965). However, the antibiotics streptomycin, griseofulvin and cyclohexamide, have sometimes been found useful (Goldberg, 1964).

During the 1930's interactions between microbes were investigated primarily in mixed or dual cultures grown on agar. Investigations using Helminthosporium sp. and other pathogens grown in the presence of bacteria, yeasts and fungi were described by Porter (1924). Subsequent research showed that antagonism in vitro was mainly attributable to mycoparasitism (one microbe parasitising another) or to the action of inhibitory substances released by the antagonistic organisms (Fawcett, 1931; Porter & Carter, 1938; Waksman, 1941; Weindling et al., 1950; Wood & Tveit, 1955; Darpoux, 1960; Barnett, 1963, 1964; Boosalis, 1964; Touzé-Soulet, 1967).

Experiments on microbial antagonism in vivo have been carried out from about 1930 onwards. Adam and Pugsley (1935) showed that symptoms of bean halo blight caused by Pseudomonas phaseolicola Burkh.

were decreased by a yellow bacterium frequently associated with the blight lesions. The symptoms of halo blight were also reduced if Pseudomonas fluorescens Mugula was applied to bean plants before inoculating with P. phaseolicola (Teliz-Ortiz & Burkholder, 1960).

The antagonistic relations between saprophytic and pathogenic bacteria are complex due in part to the ability of some plant pathogenic bacteria to survive as epiphytes on plant leaves (Crosse, 1959, 1965) so becoming resident sensu Leben (1961, 1963, 1965a). Thus populations of Pseudomonas mors prunorum Wormold on cherry trees vary independently of the amounts of disease on the trees (Crosse, 1963). The ability of pathogens to survive epiphytically may be a restricted phenomenon because although several pathogenic bacteria have been observed to become firmly established on the leaves of their natural hosts they have invariably failed to colonise leaf surfaces of non-host plants (Ercolani, 1969; Mew & Kennedy, 1971). Another problem is that antagonistic saprophytic bacteria were apparently unable to maintain themselves at an artifically produced high density on leaf surfaces (Crosse, 1965; Teliz-Ortiz, 1967). Thus in trial conditions it was necessary to use mixed bacterial suspensions with a high saprophyte/parasite ratio in order to obtain a considerable reduction in the severity of the disease (Crosse, 1965; Goodman, 1967; Scherff, 1973). However, the same ratios may not be required for disease reduction on different aerial parts of the same host. The leaf pathogen Pseudomonas glycinea Coerper may colonise the bud of soybean without the buds becoming diseased (Leben et al., 1968). This may be due to the ability of a yellow saprophytic antagonistic bacterium to compete more successfully with the pathogen on the buds than on the surface of expanded leaves (Scherff, 1973).

The nature of antagonistic interactions between bacteria is not clear. The development of fireblight symptoms in shoots of apple or pear inoculated with Erwinia amylovora (Burrill) Wimslow were reduced not only by other species and genera of bacteria, but also by an avirulent isolate of E. amylovora (Farabee & Lockwood, 1958; Goodman, 1964, 1965, 1967). These authors consider that the protection was not species specific but due to the capacity of the antagonistic bacteria to persist in significant numbers on the leaves for a period of time. Although the growth of E. amylovora was suppressed by the acid produced by the antagonist, it has been suggested that protection resulted from a host response initiated by the antagonistic bacterium (Goodman et al., 1967). This hypothesis was supported by work in which heat killed cells of bacteria protected tobacco leaves from bacterial infection (Lovrekovich & Farkas, 1965; Lozano & Sequeira, 1970). However, Chatterjee et al. (1969) suggested that protection against fireblight by the antagonistic bacteria was attributable directly to the interference of the metabolic activities of the pathogen. A bacterial interaction apparently not involving either an active host response or a direct metabolic interference also has been reported. The authors suggest that the inhibition of tumour formation is due to the exclusion of virulent strains of Agrobacterium tumefaciens (E.F. Smith & Towns.) Conn from specific attachment sites within the host tissue (Lippincott & Lippincott, 1969).

Saprophytic bacteria may also be antagonistic to pathogenic fungi. The formation of smut galls on corn leaves by Ustilago maydis (DC) Corda was inhibited by antagonistic bacteria (Bamberg, 1931) as were pustules of various cereal rusts (Levine et al., 1936;

Pon et al., 1954; Morgan, 1963). Saprophytic bacteria inhibited the infection of lettuce leaves by B. cinerea (Newhook, 1951a), tomato leaves by Alternaria solani Sor. (Darpoux et al., 1952), wheat and barley leaves by Drexlaria sorokiniana (Saco.) Subram. & Jain (Simmonds, 1947; Gayed, 1966), and Douglas fir seedlings by Melampsora medusae Thüm. (McBride, 1969). The use of high concentrations of bacteria, the addition of nutrients to the cell suspension, and conditions of relatively high humidity improved disease control (Leben, 1964; Leben & Daft, 1965), whereas in the variable conditions found in the field, especially dry atmospheric conditions, led to failure in disease reduction using bacteria previously shown to be antagonistic to several pathogens (Leben et al., 1965).

Although yeasts are common leaf epiphytes (Last & Deighton, 1965) they have rarely been observed to be antagonistic to leaf pathogens. Candida sp., a yeast which occurs in large numbers on rice leaves, halved the infection of this plant by Cochliobolus miyabeanus (Ito & Kurib.) Drechsl. (Akai & Kurumota, 1968) and both S. roseus and Torulopsis sp. reduced the infection of larch seedlings by Meria laricis Vuill. (McBride, 1970). The addition of pollen to spore suspensions of D. sorokiniana increased the extent of leaf necrosis on rye leaves (Fokkema, 1971). The addition of S. roseus and other filamentous fungi to the inoculum reduced the stimulatory effect of the pollen (Fokkema, 1973). Similar effects of S. roseus, Torulopsis sp. or Candida sp. were noted when added to Phoma betae Frank stimulated by pollen. (Warren, 1972b).

The development of plant pathogenic fungi on plant tissue can be inhibited by other fungi in several ways: (a) the active parasitism of spores and/or hyphae by fungal mycoparasites or hyperparasites (Darpoux, 1960; Barnett, 1964; Boosalis, 1964; Touzé-Soulet, 1967). (b) by non hyperparasitic fungi. A wide variety of fungi and other micro-organisms decreased the rate at which lettuce leaves were rotted by inhibiting germination and mycelial growth of Botrytis cinerea (Asthana, 1936; Newhook, 1951b; Wood, 1951). A high pH attributable to antagonists was responsible for the inhibitory effects (Newhook, 1951b). A similar pH change, caused by C. herbarum, accompanied by the production of metabolites toxic to the pathogen was thought to explain the observed control of B. cinerea on strawberries (Bhatt & Vaughan, 1963). Penicillium sp., C. herbarum and other fungi control B. cinerea on tomatoes. Good control was obtained if a period for the establishment of the saprophyte, under high humidity conditions, was allowed before inoculation with the pathogen. Among the numerous antagonistic fungi C. herbarum and Penicillium sp. had the advantage of being able to colonise leaves under much drier conditions than the other organisms tested (Newhook, 1957). C. herbarum, C. cladosporioides and A. pullulans have been observed to inhibit the germination of spores of D. sorokiniana on barley (Diem, 1969a). A similar effect observed on agar was shown to be due to nutrient competition (Diem, 1969b). The hypothesis that antagonism is attributable to nutrient competition may explain the reduction in stimulatory effect of pollen on the infection of rye by D. sorokiniana in which no difference in antagonistic properties was observed for different groups of saprophytic phylloplane micro-organisms (Fokkema, 1971, 1973). A comparable interference of the stimulatory effect of pollen by

saprophytic phylloplane fungi was observed in the infection of sugarbeet by Phoma betae (Warren, 1972a, b). Other investigations revealed that in some instances antagonism was probably due to the action of inhibitory substances produced by the antagonist. Application of spores or culture filtrates of the pathogenic fungus D. sorokiniana inhibited Septoria passerinii Sacc. on barley leaves and the formation of rust pustules Puccinia graminis on wheat leaves. This inhibition was effective when the spores or culture filtrates were added before or after inoculating with the pathogen (Morton & Peterson, 1960; Wibe & Morton, 1962; Stewart & Hill, 1965a, b). The culture filtrate of D. sorokiniana contained a non-phytotoxic substance which inhibited the germination of P. graminis uredospore and the formation of uredosori (Stewart & Hill, 1965a). Several other fungi especially Chaetomium globosum Kunze ex. Fries, Aspergillus japonicus Saito and Fusarium oxysporum Schlecht restricted the development of Puccinia penniseti Zimm. on pearl millet (Kapooria & Sinha, 1969). Rhizopus nigricans Ehrenb. decreased the numbers of lesions on potato leaves caused by Alternaria solani (E & M) Sorauer (Das & Pal, 1968). In both instances, these reductions were paralleled by the inhibition in vitro of pathogen spore germination. However, whereas A. pullulans reduced both spore germination of Alternaria zinniae Pape and the subsequent numbers of lesions formed on bean leaves, it did not inhibit spore germination in vitro. This suggested that an inhibitory substance might be produced by the leaves when influenced by the antagonistic saprophyte (van den Heuvel, 1969).

Other experiments in which leaves were protected from attack by an avirulent strain of the pathogen or by a fungus closely related to the pathogen, suggested that an inhibitory substance was formed, but no conclusive evidence was obtained (Yarwood, 1954, 1956; Day, 1957; Johnston & Huffman, 1958). However, some later experiments did provide positive proof of the formation of phytoalexin-like compounds within leaves. Two such compounds were isolated from around hypersensitive lesions on rice leaves, having been induced by an avirulent strain of Piricularia oryzae Cav. These compounds reduced the size and number of lesions (Kiyosawa & Fujimaki, 1967; Ohata & Kozaka, 1967). Hypocotyls of beans were rendered resistant to varietal pathogenic races of Colletotrichum lindemuthianum (Sacc. & Magn.) Bri. & Cav. by inoculating with Helminthosporium carbonum Ullstrup., Alternaria sp. and non pathogenic races of C. lindemuthianum. A primary protective response, as well as the formation of an unknown inhibitory substance occurred within 24 hours of inoculation with non pathogenic fungi and a second inhibitor appeared later when the tissue reacted hypersensitively (Rahe et al., 1969).

As the inhibition of a virulent strain of a pathogen by the prior inoculation of the leaf by an avirulent strain has been termed antagonism (Yarwood, 1954, 1956), the converse might be termed synergisim. Erisiphe graminis hordei Merat was only able to infect wheat plants which had been inoculated two days previously with E. graminis tritici DC. to which these plants are susceptible (Moseman & Greeley, 1964). Another example is the stimulation by Pseudomonas sp. of scale tip rot caused by Fusarium oxysporum (Bald & Solberg,1960). The only recorded instance of synergisism between phylloplane bacteria was an observed increase in the size of lesions

on ivy leaves caused by Xanthomonas hederae Arnold when this bacterium was associated with another yellow bacterium (Burkeholder & Guterman, 1932). In later work on the same disease, several associated bacteria were isolated, most of which gave no interaction with the pathogen. However one isolate did stimulate lesion development, whereas a second isolate reduced the effect of X. hederae (White & McCullock, 1934).

The successful antagonism of pathogens by saprophytic micro-organisms has been shown to be markedly influenced by the ability of a saprophyte to maintain itself at reasonably high numbers (Goodman, 1967), especially under adverse field conditions (Leben et al., 1965). Thus an antagonistic saprophyte which can colonise the aerial surfaces of plants should be able to effectively reduce the incidence of a particular disease. This has been shown to be true for the bacteria antagonistic to Ps. glycinea colonizing soybean buds (Scherff, 1973).

Many studies have been done on the incidence of saprophytes on the phylloplane, especially in relation to plant age and the time of the year. These have been well reviewed (Last & Deighton, 1965; Sinha, 1965; Leben, 1965a; Hudson, 1968; Last & Warren, 1972). However few reports have related the incidence of micro-organisms to their activity on the leaf surface. Many of the fungi isolated may have been present only in the form of spores, as few have been observed in filamentous form on the surface of leaves, especially early in the growing season (Dickinson, 1967), and mycelial development may well only occur from the time when leaves show evidence of senescence (Hollowman, 1967; Bainbridge & Dickinson, 1972). The division of fungal species present on the leaf into three groups on the basis of a temporal sequence was discussed by Hogg & Hudson

(1966). Among the primary colonisers which they listed were Alternaria sp., Aureobasidium pullulans, Botrytis cinerea and Cladosporium cladosporioides. Although the activity of these primary colonisers has been confirmed (for example the active sporulation of Cladosporium sp. has been observed on the leaf surface (Dickinson, 1965)), observations by other workers indicated that this was not a uniform group in which all organisms grew together, but that competition largely determined the period of active growth (Pugh & Buckley, 1971a).

These variations in activity were not restricted to the filamentous fungi as it has been shown that white yeasts and pink yeasts may compete (Last, 1955a) and that although S. roseus was unable to colonise aseptic larch seedlings, under the conditions of the experiment, the percentage survival of yeast cells was found to vary with leaf age (McBride, 1970). Bacterial colonisation of seedling leaves is due not only to multiplication of the bacterial cells, but also to the migration of bacteria from infested seeds (Leben, 1963). The extent of this migration is dependent on the humidity of the atmosphere around the plant (Leben & Daft, 1966). The quantitative study of the colonisation of plants from a known inoculum concentration showed that Pseudomonas glycinea could multiply on soybean buds although observations on individual buds varied markedly (Leben et al., 1968). A later study by other workers showed that numbers of Ps. glycinea increased a thousandfold within seven days of inoculation onto soybean leaves. From that time onward the increase in numbers was less marked on cultivars susceptible to this bacterium and the population decreased on resistant cultivars (Mew & Kennedy, 1971).

Saprophytic phylloplane micro-organisms not only show variation in activity, but also a non random distribution over the surface of the leaf. The depressions overlying the anticlinal cell walls of epidermal cells have been observed to support larger numbers of yeasts (Last, 1955a) and bacteria (Ruinen, 1961; McBride, 1970) than other areas of the leaf lamina. These depressions have also been observed to be important infection sites for pathogenic fungi (Preece et al., 1967). High numbers of phylloplane micro-organisms have been observed to be associated with the vascular areas of leaves. Numerous yeast cells were observed along the vascular strand of pea leaves (Dickinson, 1967) and the localisation of A. pullulans on veinal tissue has been observed on lime (Pesante, 1963) and sycamore (Pugh & Buckley, 1971a). Similarly, cells of Pseudomonas syringae van Hall were concentrated above veins on soybean leaves (Leben et al., 1970).

METHODS

Much of the examination of possible antagonistic mechanisms between saprophytes and plant pathogens has used *in vitro* methods, particularly agar plate techniques. The bulk of the earlier work in this field was reviewed by Waksman (1941). Other methods have included the use of pairs of organisms supported in fluid droplets on glass slides (Bier, 1965; McBride, 1970). The validity of such methods in the study of microbial antagonism has been questioned (Huber & Watson, 1966). The observed discrepancy between the ability of an organism to inhibit the growth of another in culture and its ability to function as an antagonist on the plant surface has also been recognised (Bhatt & Vaughan, 1963; Akai & Kuromota, 1968; van den Heuvel, 1970), although it has been assumed that effects *in vitro* were still indicative of potential antagonistic capacity *in vivo* (van den Heuvel, 1970). Furthermore, the relatively less complex environment of the culture plate may enable some explanations to be obtained for interactions observed on the surface of leaves. One example is the observed antagonism of *Drexlaria sorokiniana* on the leaves of barley by *Cladosporium* sp. and *Aureobasidium pullulans* (Diem, 1969a) which plate culture techniques showed to be due to nutrient competition (Diem, 1969b).

The ability of the antagonistic saprophytic organisms to establish and maintain themselves on leaf surfaces as a prerequisite for the long term inhibition of pathogens was stressed by Newhook (1957), Leben *et al.* (1965) and Goodman (1967). Therefore it is not surprising that the cultural techniques used for studying the colonisation of the leaf surface, discussed earlier (p.27), have

been used in studies on microbial antagonism, although in a few instances the direct effects of the reduction in the appearance of a particular disease symptom, especially aggressive lesions, has been utilised (van den Heuvel, 1969; Warren, 1972b; Fokkema, 1973). Fungi have also been observed directly and spore germination and germ tube growth assessed (Blakeman, 1968; McBride, 1970). This has involved the decolourisation of leaf tissue before staining and subsequent microscopic examination. The bleaching of leaves using chlorine gas (Janes, 1962) has been used frequently in studies on phylloplane micro-organisms (Blakeman, 1968; McBride, 1970; van den Heuvel, 1970; Fokkema, 1971; Skidmore & Dickinson, 1973) hoping that it causes minimal disturbance to the spatial arrangement of these organisms on leaves, although some movement may occur (Dickinson, 1967). However, when parallel studies were done comparing the above technique and scanning electron micrographs, the possible defects of leaf bleaching and subsequent staining were not substantiated (Pugh & Buckley, 1971a).

The initial colonisation of leaves by micro-organisms has been studied using washing techniques following the application of a known inoculum to glasshouse grown plants (van den Heuvel, 1969), aseptic seedlings in flask culture (McBride, 1970) or seedlings in partial aseptic conditions under thin film isolators (Leben, 1963; Leben et al., 1968; Mew & Kennedy, 1971). The results obtained were variable, for in some instances the micro-organisms failed to maintain themselves on the leaves. In all instances the inconsistencies were ascribed to the effects of the varying environmental conditions. It seemed preferable to adopt methods using isolated pieces of plant tissue which were sufficiently small to enable the surrounding

environment to be controlled more readily. Leaf discs have been used in some studies (Blakeman & Dickinson, 1965; Pukayastha & Deverall, 1965; Skidmore & Dickinson, 1973), but many investigations on leaf pathogens have been done using detached leaves (Samborski & Forsyth, 1960; Channon, 1967; Malcolmson, 1969; Pegg & Mence, 1970; Sargent et al., 1973). Both techniques can be critisised because detached tissues are essentially senescing even though senescence may be delayed to some extent by the use of compounds such as benzomydazole (Wolfe, 1965). The availability of nutrients on leaf discs is affected by leakage from ruptured cells around the cut edges and an increase in nutrients, especially carbohydrates, has been observed in leaves detached from a parent plant (Cram, 1972). However, if whole leaves are detached and the petioles treated with a suitable rooting hormone, these leaves will form roots in a nutrient medium (Gregory & Samantarai, 1950) to give a compact plant system which could readily provide uniform material suitable for physiological experiments (Spence et al., 1972). If leaves from plants grown in glasshouse conditions, in which few microbes are found on the leaves (Leben & Daft, 1966), were detached and surface sterilised using a reasonably efficient procedure, (Williams et al., 1966), it should be possible to use such compact plant systems to study microbial colonisation and interactions on the phylloplane under more readily controllable environmental conditions.

CULTURE AND PREPARATION OF MICROBIAL INOCULA

Suspensions of S. roseus and C. cladosporioides were produced by the methods described on page 61,65. Uredospores of P. antirrhini were obtained by the methods of Williams et al. (1966), which enabled contaminant free uredospores to be obtained. Leaves of A. Nanum were inoculated with uredospores applied with a sterile paint brush. These plants were then wetted with atomised distilled water, kept in the dark for 48 hours at 10°C, and returned to the glasshouse. Four days later the infected leaves were detached and placed in a sealed dark container for 15 minutes to encourage stomatal closure (Meidner & Mansfield, 1968), so as to minimise deleterious effects of the surface sterilisation procedure upon the leaves. The leaves were then placed in a hypochlorite solution (3% available chlorine) for three minutes before washing three times in sterile distilled water, and transfering to petri dishes containing tenth concentration Olson solution (Olson, 1944) in 0.75% agar. The petioles were firmly embedded in the agar and the leaves placed in conditions of 16 hours light at 18°C (leaf temperature) followed by 8 hours darkness at 10°C. Leaf temperature was monitored using a thermocouple embedded into the epidermis of a sample leaf (Waggoner & Shaw, 1952) contained in a petri dish. After 10 days uredosori began to appear and 7 days later uredospores were collected and suspended in sterile deionised water.

EFFECT OF ANTIRRHINUM LEAF LEACHATES ON GROWTH AND DEVELOPMENT OF P. ANTIRRHINI, C. CLADOSPORIOIDES AND S. ROSEUS

Leaf leachates of both cultivars of antirrhinum were obtained by the methods described previously (p. 102). Leachates of leaves having a leaf plastochron index (LPI) of 1.5 and LPI -0.5 were concentrated to 2K (Kovac's concentration, p. 102)(Kovacs & Szeöke, 1957). Spore suspensions of filamentous fungi were prepared as described above (p. 61) to give spore concentrations of 25×10^4 ml^{-1}. Aliquots (0.02 ml) of spore suspensions were added to 0.02 ml leachate, giving a final leachate concentration of 1 K, on a sterile glass slide which was placed in a humidity chamber. These chambers were maintained at 25°C for C. cladosporioides and 10°C for P. antirrhini, the optimal germination temperatures for each fungus respectively. As the spore densities attained were less than 5000 cm^{-2} no adverse effect due to spore concentration occurred (p. 69,73).

Suspensions of S. roseus were prepared as described previously (p. 65) to give a cell concentration of 25×10^4 ml^{-1}. 0.02 aliquots were mixed with 0.02 ml of leachate, giving a final concentration of 1 K, on sterile cover slips (18 x 18 mm), which were placed in humidity chambers at 18°C (optimal growth temperature). Numbers of viable cells in the suspension were assessed by dilution plate counts and the sizes of 20 cells were measured using an ocular micrometer on a phase contrast microscope, enabling an estimate of the total volume of cells to be ascertained. This was necessary as changes in size of S. roseus cells had been observed in preliminary experiments.

Five replicates were set up for each leachate solution in combination with each microbe, for each incubation period. Parallel sets of five replicates of controls were set up using 0.02 ml sterile distilled water in place of the leachate solution. The saprophytes were sampled after 24 and 48 hours and the pathogen after 48 hours only. This was because the effect of exogenous compounds on these saprophytes had been shown to increase their rate of growth (p. 72) whereas no such effect was observed with P. antirrhini whose maximum germination occurred only after 48 hours incubation (p. 70).

The filamentous fungi were sampled by mixing a drop of 2% Trypan blue in lactophenol with the sample droplet on the slide and applying a coverslip. Two hundred spores per slide were examined for germination using the criteria described above (p. 60). All spores in a field of view were assessed. The fields were chosen by random number tables and the micrometer stage on the microscope. 20 germ tube lengths chosen randomly were measured using an ocular micrometer.

The increase in cell numbers of S. roseus was determined by placing each coverslip, bearing a droplet, into a sterile 50 ml conical flask containing 10 ml of a sterile solution of 0.01% Tween 80. These flasks were shaken for 30 minutes on a wrist action shaker and from the resulting suspension a dilution series was prepared. Aliquots (0.1 ml) were surface plated onto malt agar and incubated at 18°C for 5 days before counting. Samples of each suspension were also observed by phase contrast microscopy and the size of 20 cells per sample measured using an ocular micrometer.

COLONISATION OF ANTIRRHINUM LEAVES BY S. ROSEUS AND C. CLADOSPORIOIDES

Plants of A. Nanum and A. Fi hybrid were grown from seed using the procedure described previously (p.33). Cell and spore suspensions of S. roseus and C. cladosporioides were prepared at concentrations twice as great as finally required. Equal volumes of the suspensions of each organism were then placed in a sterile vial and mixed thoroughly on a wrist action shaker. Single culture suspensions were prepared using equal volumes of microbial suspension and sterile water. The resulting concentrations were checked using a haemocytometer. This procedure enabled equal numbers of cells of one micro-organism to be applied in a set volume using either single or mixed cultures.

Before commencing any experiment, the test plants were monitored for chance phylloplane microbes (p. 33). Then leaves of LPI 1.5 were selected and marked by applying a small coloured self-adhesive disc to the leaf petiole. Different colours were used to indicate which of the three suspensions (1. S. roseus alone, 2. C. cladosporioides alone, 3. S. roseus + C. cladosporioides) had been used to inoculate a particular leaf. Aliquots (0.05 ml) of microbial suspension were inoculated onto the upper surface of the leaf using a microsyringe. The inoculum did not remain as a discrete droplet, but spread out over the leaf surface, which only remained obviously wet for about one hour. At this time five leaves were removed from each single culture treatment and 10 leaves were removed which had been inoculated with the mixed culture. This was done to ascertain the recovery of S. roseus from the leaves and so provide a well defined base line for future changes of cell numbers and volume, for both single and mixed cultures.

The leaves bearing C. cladosporioides alone or in mixed culture were examined to ascertain if any pregerminated spores had been applied. Leaves were sampled during the three week period after inoculation.

The sampling procedure for S. roseus was as follows. Five leaves inoculated with S. roseus alone and five leaves inoculated with S. roseus and C. cladosporioides were removed from the plant using sterile scissors and forceps. Each leaf was placed in a 50 ml conical flask containing 10 ml of a sterile solution of 0.01% Tween 80. Each flask was corked and shaken vigorously on a wrist action shaker for 30 minutes at room temperature. Dilution series were prepared from the resulting suspensions. Aliquots (0.1 ml) were surface plated onto three replicate plates of malt agar (Oxoid) at each dilution. These plates were incubated at 18°C for five days before counting. Samples from each suspension were also examined by phase contrast microscopy and the dimensions of 20 cells, selected randomly (p.139), were measured using a calibrated ocular micrometer.

At each sampling occasion, numbers of S. roseus present on leaves were also monitored using the sporefall technique (Last, 1955a). Five leaves inoculated with S. roseus alone and five leaves inoculated with S. roseus and C. cladosporioides were sampled using this technique.

The colonisation of leaves by C. cladosporioides was assessed in terms of spore germination and germ tube length. Five leaves inoculated with C. cladosporioides alone and five leaves inoculated with mixed cultures were assessed at each time period. These leaves were decolourised by chlorine gas (Janes, 1962) and then exposed to ammonia vapour to neutralise any residual chlorine. The leaves were placed on slides, stained with 1% Trypan blue in lactophenol and left for 24 hours

to allow the stain to be absorbed by the spores. These leaf preparations remained in good order for about one month before the stain showed signs of fading. Some preliminary investigations suggested that different areas of leaves might affect spores in different ways (Pugh & Buckley, 1971a). To make allowance for this possible difference, spores were assessed on the leaf midrib and on an area of lamina free from readily visible vascular tissue. 200 spores selected at random (p.139) were assessed for germination, on each area of the leaf. The lengths of 30 germ tubes were also measured in these two areas, using the methods described in Section 1 (p.63). For midrib studies one axis of the stage micrometer was locked and random numbers applied to the other axis only.

Some preliminary experiments were done using plants growing in the glasshouse in 16 hour days at approximately 18°C and a night temperature of 10-12°C. These experiments proved unsatisfactory, since numbers of yeasts decreased rapidly and although few spores of C. cladosporioides germinated, germ tube growth was observed only within the first day after inoculation. The daytime humidity, measured by Assman psychrometer, was found to be 75%, whereas spores of C. cladosporioides require a humidity in excess of 94% R.H. for germination to occur (p.70). Moreover, Leben and Daft (1966) considered that the low numbers of micro-organisms isolated from glasshouse plants and in some field experiments were due to the low humidities prevailing, and so it was considered that the failure of the glasshouse experiments described above could be attributed to the low humidity. Subsequent experiments were done in an environment room set to give conditions of 16 hour days at 18°C and 10°C nights at a relative humidity of approximately 95% R.H. The plants were inoculated

and sampled using the above procedures (p.141).

COLONISATION OF ROOTED DETACHED ANTIRRHINUM LEAVES BY S. ROSEUS AND C. CLADOSPORIOIDES

Even though all possible precautions were taken during leaf colonisation experiments, results were variable and so attempts were made to devise some system which might enable patterns of colonisation and interactions between these micro-organisms to be discerned more readily. Although many workers have used detached leaves (p.136), this form of plant culture has disadvantages, especially those resulting from the gross physiological disorder of the detached tissue (Yarwood, 1946), but these may be obviated by the use of rooted detached leaves (p.136). Although the leaves used in these experiments were of a defined physiological age in terms of plastochron index when detached from plants, subsequently little leaf growth occurred and so their precise physiologic age (measured in plastochrons) during an experiment was not known. Therefore in experiments on the colonisation and microbial interactions on detached leaves the plastochron index is used merely to define the physiologic age at which leaves were removed from the plant and leaves are referred to as old (detached at LPI 1.5) or young (detached at LPI -0.5).

After a series of tests using different hormone treatments to enhance possible rooting responses the following technique was found to be successful and convenient for the preparation of surface sterilised rooted detached leaves.

Intact leaves of the required physiologic age were removed from plants and surface sterilised using the procedure described above (p. 137). After washing in sterile deionised water, the ends of the leaf petioles were stood in a sterile solution of Naphthaline acetic acid (NAA) at a concentration of 100 ppm for 36 hours. This solution was prepared by dissolving a known weight of NAA in 95% ethanol and then diluting with deionised water to give 100 ppm. Sterilisation was by autoclaving for 15 minutes at 121°C. After removal from this solution, the leaf petioles were firmly pressed into a mineral salts agar medium (p.137) in a petri dish. These petri dishes were placed under a light bank (6 x 65 watt warm white fluoresent tubes supplemented by 500 watts of incandesent tungsten illumination), set to give 16 hour days at 18°C (leaf temperature within the petri dish measured by a thermocouple embedded into the leaf) and 10°C nights in a cold room. After three days root initials could be seen on the petioles and after 10 days roots began to grow (Plate 13). This rooting response was found in all antirrhinum leaves during their logarithmic phase of growth (leaf plastochron index -0.45 to 1.68).

Although more or less uniform leaf material in a physiologically normal state could be produced, the environmental conditions within the petri dishes might still be unsuitable for the development of phylloplane micro-organisms (Leben & Daft, 1966). Although leaf temperature could be measured reasonably conveniently using thermocouples (Waggoner & Shaw, 1952), the measurement of humidity within the confines of a 100 mm petri dish posed problems. Although very small droplet psycrometers have been developed (Cotton, 1969) the designs would not permit them to be inserted into a petri dish

Plate 13. Rooted detached leaf of A. Nanum growing in a nutrient medium.

conditions. As it was likely that the humidity at the leaf surface might be higher than the surrounding atmosphere, then conditions prevailing at the leaf surface should allow C. cladosporioides to germinate (p.70) and yeasts to multiply (Leben & Daft, 1966).

Samples of the detached leaves were checked for microbial contaminants both before experiments commenced and at intervals during the experiments. Nutrient agar (Oxoid) plus 0.75% Yeast extract (Difco) was used in addition to malt agar to allow any bacteria present to grow more readily. The detached leaves were inoculated with suspensions of S. roseus and C. cladosporioides 10 days after they had been placed on the mineral salts medium at which time roots had also appeared. The method of obtaining uniform suspensions of micro-organisms, the inoculation, and sampling technique were the same as used in the tests on intact plants described above. Leaves were sampled after 1, 2, 4, 5, 6, 7, 14 and 21 days. Originally it had not been intended to isolate from the leaves on days 5 and 6, but early experiments showed that the percentage germination of conidia of C. cladosporioides decreased between days 4 and 7 and so the intervening days were sampled to try to locate the exact time that this possible decrease began.

Experiments on attached leaves and the first experiments on detached leaves were done using leaves of LPI 1.5. When younger leaves LPI -0.5 were used it was found that inoculation droplets of 0.05 ml would not stay on the leaf and so it was necessary to reduce the volume of inoculum. This in itself was simple but in order to compare leaves of different ages directly and to avoid effects due to differences in microbial population on the leaf, some means of ensuring nearly equal numbers of organisms per unit area of leaf was required.

The 0.05 ml droplets spread out over part of the surface of the older leaves and it would have been wrong to have assumed that the area of a younger leaf covered by a 0.02 ml droplet which had spread out would have been in direct proportion to the different volume of the droplets.

A small preliminary investigation was done to find the surface area covered by the different volumes of inoculum on the surface of different ages of leaves. This experiment utilised a slight modification of a method used to study the retention of droplets on leaves for pesticide trials (Amsden & Lewins, 1966). An aqueous solution of crystal violet (3%) was used and 0.05 ml droplets placed on 30 leaves (LPI 1.5) of A. Nanum and of A. Fi hybrid. Similarly 0.02 ml aliquots were placed on 30 younger leaves (LPI -0.5) of each cultivar of antirrhinum. This operation was done in the laboratory where the atmosphere was reasonably dry. After a short period the droplets had dried, staining the area of the leaf which had been covered. These stained parts of the leaves were cut out under a dissecting microscope and the areas of the stained leaf pieces, estimated on a leaf area machine (Hayashi Denko Manufacturing Company, Japan) (Table 20). The mean value for the older leaves of both cultivars of antirrhinum was 2.45 cm^{-2} and for younger leaves 0.98 cm^{-2}. There was no significant difference ($p = .05$) between the mean values of each cultivar within each age class of leaves. As 10^5 organisms had been applied to the older leaves giving 4081 cm^{-2} then 4×10^3 needed to be applied to the younger leaves in a 0.02 ml aliquot to obtain the same number of organisms per square centimetre. Thus this procedure was adopted.

Table 20 Surface area of leaves of A. Nanum and A. Fi hybrid covered by droplets of known volume of a crystal violet solution

Leaf age (LPI)	Cultivar	Droplet Volume (ml)	Mean area covered (cm^{-2})	95% confidence limits about the mean
1.5	Nanum	0.05	2.38	0.15
	Fi hybrid	0.05	2.52	0.18
-0.5	Nanum	0.02	0.92	0.17
	Fi hybrid	0.02	1.04	0.18

INTERACTIONS BETWEEN P. ANTIRRHINI, C. CLADOSPORIOIDES AND S. ROSEUS

IN VITRO

This series of experiments was done using different combinations of micro-organisms supported in droplets of deionised water on glass slides or coverslips. The suspensions of micro-organisms were prepared as described previously (p.138) and 0.02 ml aliquots of each suspension were placed on sterile slides or coverslips to give the following inocula:-

1) P. antirrhini alone
2) C. cladosporioides alone
3) S. roseus alone
4) P. antirrhini + C. cladosporioides
5) P. antirrhini + S. roseus
6) P. antirrhini + C. cladosporioides + S. roseus
7) C. cladosporioides + S. roseus

All droplets were made up to a final volume of 0.06 ml with sterile deionised water, so that in all treatments the droplet volume was equal. In treatment (3) S. roseus alone was set up on sterile coverslips (18 x 18 mm) and for (5), (6) and (7) parallel series were prepared on coverslips and glass slides thus enabling the observations on S. roseus to be made using the washing technique detailed previously (p. 141) while the slide cultures were used for the examination of C. cladosporioides and P. antirrhini by direct microscopic procedures (p. 141). Five replicates of each treatment (or parallel set) were used. As P. antirrhini has been shown to have a very exact temperature requirement for spore germination (p. 68), it was necessary to use this optimum temperature for germination to ascertain whether any

effects which might be observed were due to microbial interaction rather than the effects of the prevailing physical conditions. All treatments were incubated in humidity chambers at 10°C for 48 hours in darkness before being observed.

2) ON ROOTED DETACHED ANTIRRHINUM LEAVES

Many workers investigating the possible antagonisms of saprophytes towards pathogens have used techniques in which the saprophytes were applied to the leaf surface some hours or days prior to the pathogen. This process might allow the saprophytes to establish themselves, but might also trigger in some way a resistance mechanism in the host or predispose the leaf to infection by the pathogen. A review of the work on predisposition and immunisation of leaves is given by Matta (1971). It was the aim of these experiments to observe if any interactions occurred between these three micro-organisms in the environment of the leaf surface rather than a situation in which the reactions of the leaf alone formed the only observable interaction with the pathogen. Therefore simultaneous inoculation of saprophytes and pathogens was used. Leaves of LPI 1.5 of both cultivars of antirrhinum were used because the results for the colonisation experiments were less variable on leaves of this age and thus there might be less chance of experimental variability masking interactions.

Parallel experiments were set up using two different sets of environmental conditions. In one the conditions were optimal for the germination of uredospores of P. antirrhini (48 hours darkness, RH 98-100%, 10°C). In the other set of experiments more natural conditions of alternating light and dark were used.

The light bank was set to give 16 hour days at a leaf temperature of 18°C and an 8 hour night at 10°C. The humidities were 95% and 98% R.H. respectively. This experiment was run for 6 complete days so that the leaves were subjected to a total dark period of 48 hours at 10°C which should have allowed the uredospores of P. antirrhini to germinate as the light inhibition of germination has been shown to be reversible (p. 68). In order to maintain these humidity conditions, which were slightly higher than in the experiments on the colonisation of leaves by saprophytes, the petri dishes were supported on wire mesh over trays of water. The whole trays were enclosed within polythene bags which were sealed. The sampling procedures adopted at the end of the incubation periods were as follows. S. roseus and C. cladosporioides were assessed by the procedures used in the colonisation experiment described above (p. 141). P. antirrhini was assessed in much the same way as C. cladosporioides excepting that only one random sample over the whole leaf area was done for spore germination and for germ tube length, as no effect due to different locations on the leaf was discernable in preliminary experiments. An assessment of the germinated uredospores whose germ tubes had penetrated the leaf either through the stomata or by the formation of appresoria and infection pegs pushed through the upper epidermis, was also made.

RESULTS

EFFECT OF ANTIRRHINUM LEAF LEACHATES ON SOME MICRO-ORGANISMS

Statistical analysis of the data on spore germination of P. antirrhini and C. cladosporioides was done using 'X^2' tests (McCallan & Wilcoxon, 1932) and spore germ tube data were analysed using the Mann-Whitney 'U' test (Siegel, 1956). The significant differences ($p = .05$) between treatments are displayed as triangular matrices for the germ tube length data and also for the germination data on P. antirrhini.

1) P. ANTIRRHINI

It is evident that leachates from young and old leaves of both cultivars of antirrhinum significantly ($p = .05$) reduced P. antirrhini uredospore germination and germ tube growth and that this reduction was significantly greater in A. Fi hybrid leachates (Fig. 23). Any differences in the response of P. antirrhini to leachates from different aged leaves of the same cultivar of antirrhinum were not significant. (Table 21)

2) C. CLADOSPORIOIDES

The percentage germination of conidia was significantly greater ($p = .05$) than the water control in all leachate solutions after 24 hours, but after 48 hours no significant differences between treatments were observed (Table 22). Germ tube lengths were significantly greater in all leachate solutions used after either incubation period (Table 23; Figs. 24, 25). Also germ tube growth was significantly greater in leachates from older leaves after both

Table 21 P. antirrhini: Germination and germ tube growth in antirrhinum leaf leachate solutions after incubation for 48 hours at 10°C

	Treatment	Germination %	Median Germ tube length (µm)
	H_2O Control	94.6	490.5
Cultivar	Leaf age (LPI)		
A. Fi hybrid	1.5	4.4	77.5
	-0.5	5.8	89.2
A. Nanum	1.5	74.3	159.4
	-0.5	78.2	194.2

Figure 23 P. antirrhini: Matrix of significant differences (p = .05) in germination and germ tube growth in Table 21

	C				
FO	+				
FY	+	-			
NO	+	+	+		
NY	+	+	+	-	
	C	FO	FY	NO	NY

Key:

C = Water control
FO = A. Fi hybrid: older leaves LPI 1.5
FY = A. Fi hybrid: younger leaves LPI -0.5
NO = A. Nanum: older leaves LPI 1.5
NY = A. Nanum: younger leaves LPI -0.5
\+ = Significant difference (p = .05) 'X^2' test for germination data and 'U' tests for germ tube length data

Table 22 C. cladosporioides: Spore germination in antirrhinum leaf leachate solutions after incubation for 24 or 48 hours at 25°C.

	Treatment	Percentage Germination	
		24 hrs.	48 hrs.
	H_2O Control	29.0	54.7
Cultivar	Leaf age (LPI)		
A. Fi hybrid	1.5	60.7 *	70.3
	-0.5	54.3 *	62.1
A. Nanum	1.5	63.1 *	76.9
	-0.5	57.6 *	63.5

* Significantly different (p = .05) from control

Table 23 C. cladosporioides: Spore germ tube growth in antirrhinum leaf leachate solutions after 24 or 48 hours incubation at 25°C.

	Treatment	Median germ tube length (µm)	
		24 hrs.	48 hrs.
	H_2O Control	20.3	50.0
Cultivar	Leaf age (LPI)		
A. Fi hybrid	1.5	68.3	242.9
	-0.5	54.6	96.2
A. Nanum	1.5	99.0	347.1
	-0.5	55.1	114.6

Figure 24/25 Significant differences (p = .05) in germ tube length after incubation in antirrhinum leaf leachate solutions

Fig. 24
24 hours incubation

	C	FO	FY	NO	NY
C					
FO	+				
FY	+	-			
NO	+	-	+		
NY	+	-	-	+	

Fig. 25
48 hours incubation

	C	FO	FY	NO	NY
C					
FO	+				
FY	+	+			
NO	+	+	+		
NY	+	+	-	+	

Key:

C = Water Control
FO = A. Fi hybrid: older leaves LPI 1.5
FY = A. Fi hybrid: younger leaves LPI -0.5
NO = A. Nanum: older leaves LPI 1.5
NY = A. Nanum: younger leaves LPI -0.5
\+ = Significant difference (p = .05) 'U' test

Table 24 S. roseus: Increase in total volume of cells incubated into antirrhinum leaf leachate solutions and incubated for 24 or 48 hours at 18°C

	Treatment	Factor for increase in total volume of cells	
		24 hrs.	48 hrs.
	H_2O Control	2.03	4.23
Cultivar	Leaf age (LPI)		
A. Fi hybrid	1.5	4.69	28.57 *
	-0.5	4.14	20.47 *
A. Nanum	1.5	4.56	36.82 *
	-0.5	4.23	26.32 *

* Significantly greater (p = .05) than control

24 and 48 hours, but after the latter time interval germ tube growth in leachates from older leaves (LPI 1.5) of A. Nanum was significantly greater than in leachates from A. Fi hybrid leaves of the same age. The observed differences between younger leaves of both cultivars were not significant.

3) S. ROSEUS

The increase in total volume of cells was significantly greater ($p = .05$) than the control in all the leachate solutions after 48 hours but not after 24 hours. However, there was no significant difference in the increase in total volume of cells between any of the leachate solutions used. (Table 24)

COLONISATION OF ANTIRRHINUM LEAVES

The observation in replicated samples in these experiments were characterised by a high degree of variability and frequent zero values for either the recovery of S. roseus or the germination of C. cladosporioides on inoculated leaves. The results for one experiment on each cultivar of antirrhinum are presented here in terms of means and ranges only in order that any overall patterns might be discerned. The remaining results, which are similar, are given in Appendix 1.

1) S. ROSEUS

The total volume of cells of S. roseus increased over the 21 day period of the experiment in all treatments (Table 25). The values of the means were lower in the isolations from leaves of both cultivars of antirrhinum inoculated with S. roseus together with C. cladosporioides. No colonies of S. roseus were isolated by the

Table 25 S. roseus: Volume of cells per leaf of A. Nanum and A. Fi hybrid isolated by leaf washing

CULTIVAR	TREATMENT	MEASURE	Volume of cells ($\mu m^3 \times 10^3$) per leaf INCUBATION PERIOD (DAYS) 0	1	2	4	7	14	21
A. Nanum	S. roseus alone	Mean	197.8	2983.2	2635.2	5532	1036.8	10476	20323
		Min.	129.6	201.6	316.8	0	0	58.32	0
		Max.	247.2	12770	45516	35424	3600	52212	117072
	S. roseus + C. cladosporioides	Mean	183.4	1039.2	3333.6	4903.2	10699	7308	9292.8
		Min.	152.9	352.8	98.4	0	0	0	0
		Max.	288.96	4300.8	126000	27360	65520	35760	52416
A. Fi hybrid	S. roseus alone	Mean	414	3192	3048	7056	6168	24576	14088
		Min.	175.2	297.6	95.6	24	0	213.6	0
		Max.	492	1344	91200	38880	21024	127200	80640
	S. roseus + C. cladosporioides	Mean	381.6	1051.2	1500	4418	1420.8	3528	7776
		Min.	213.6	511.2	171.6	331.2	0	0	0
		Max.	8328	6936	11808	17280	15336	22728	45360

sporefall technique from any of the inoculated leaves.

2) C. CLADOSPORIOIDES

The values of the means and maxima of percentage spore germination from the midrib area of the leaves of both cultivars increased to a peak in the four day sample and then decreased (Fig. 26). No such pattern was observed in the leaf lamina data. The overall germination on both areas of the leaves was similar. Also spore germination in mixed cultures with S. roseus was similar to that observed for C. cladosporioides alone.

Mean germ tube length increased with time and after 21 days samples from the leaf midrib had larger mean values than samples from the leaf lamina (Fig. 27). Also at this time mean germ tube lengths on A. Nanum were greater than those from A. Fi hybrid. Also, S. roseus reduced the length of germ tubes relative to the control of C. cladosporioides incubated alone on leaves.

All these points may be indicators of possible effects, but should be treated with reservation owing to the variable nature of the data obtained.

COLONISATION OF ROOTED DETACHED LEAVES

There was variability between data from replicate samples obtained from these experiments, but this was considerably smaller than that found in the experiments on attached leaves. The use of statistical procedures enabled some degree of confidence to be placed on observed differences. Advice on the handling of the data obtained and the statistical procedures used was obtained from Mr. E. Renshaw of Edinburgh University Department of Statistics. It was impractical

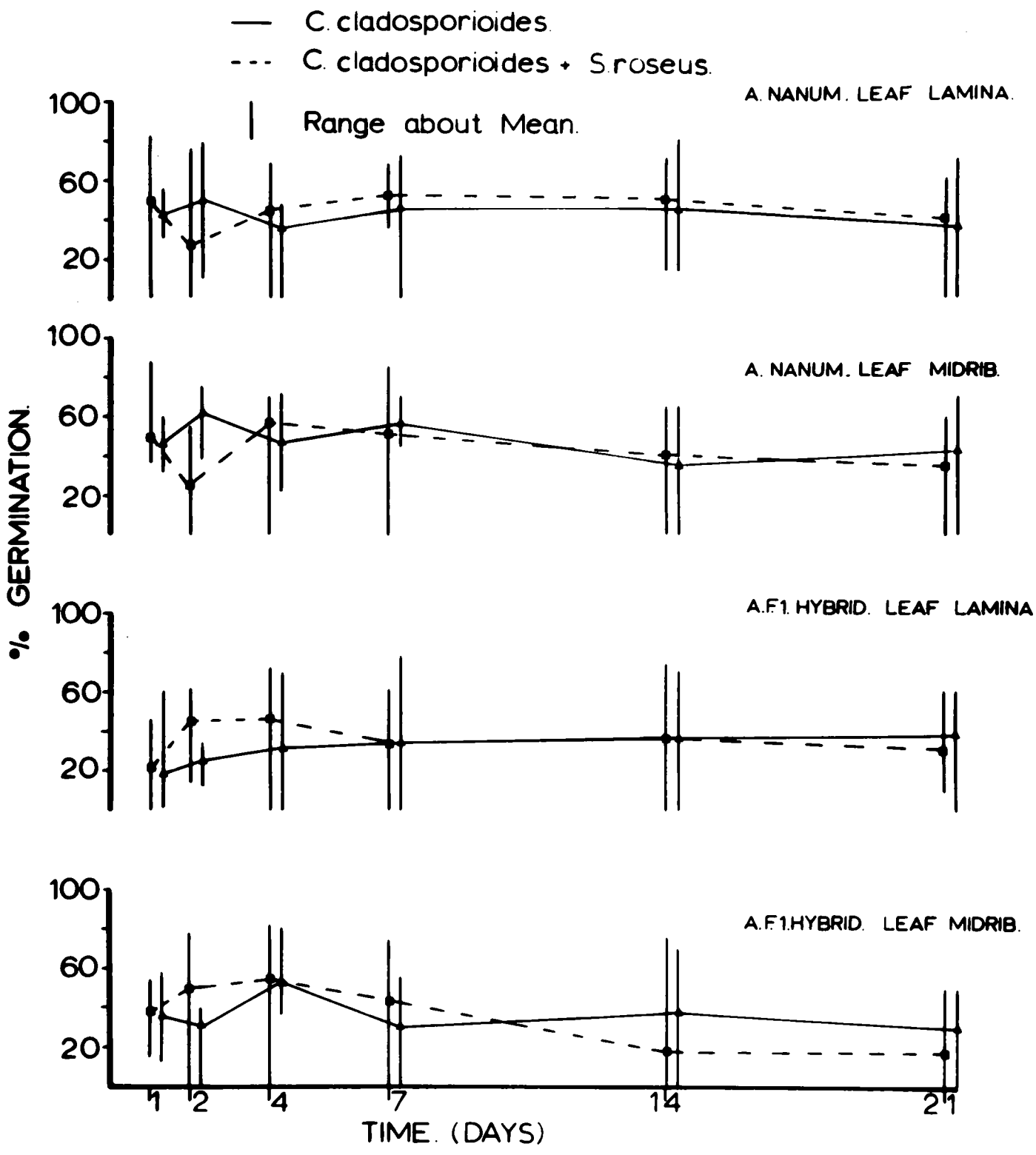


Fig. 26 C. cladosporioides: Spore germination on attached leaves of A. Nanum and A. Fi hybrid at different times after inoculation

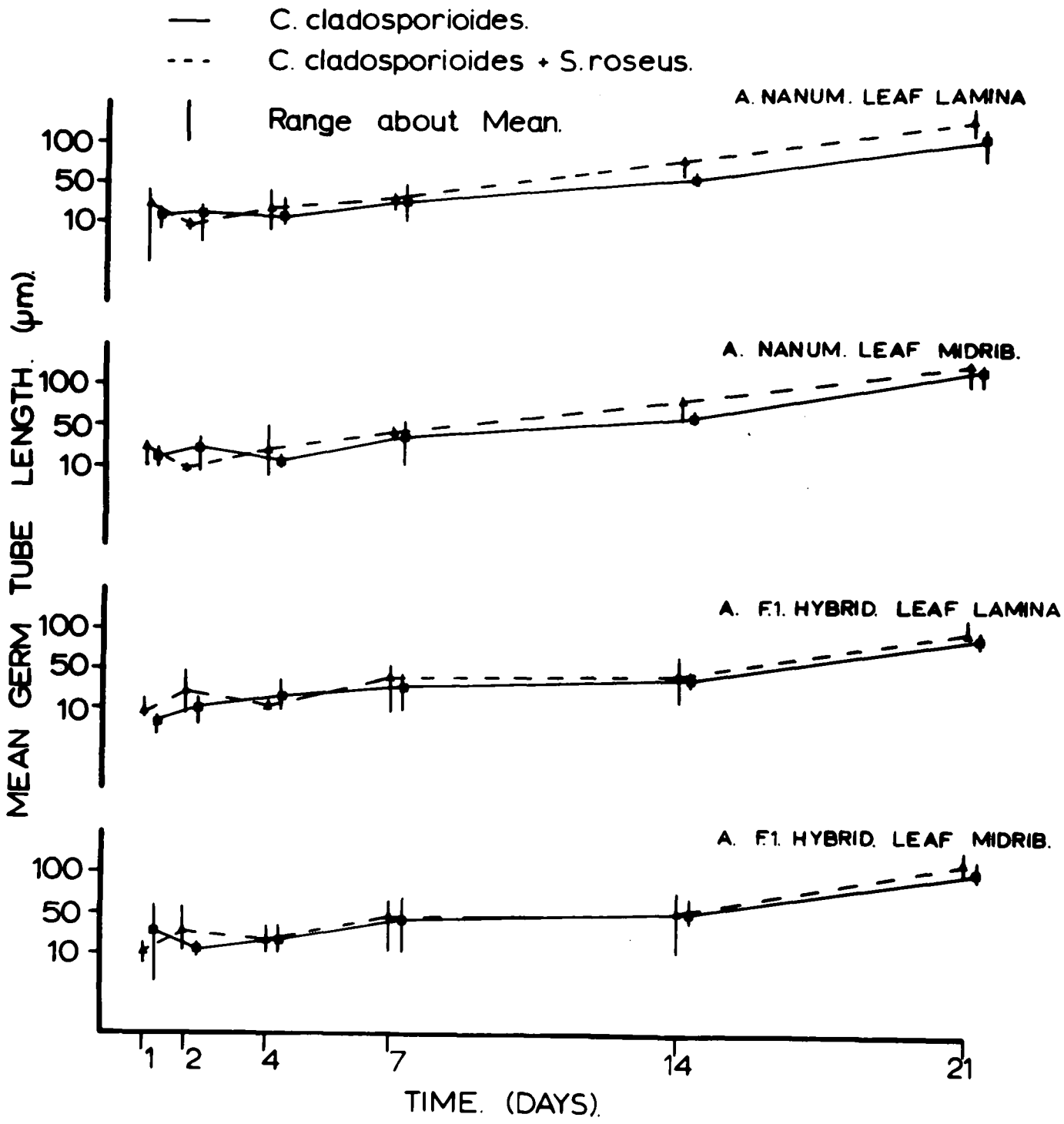


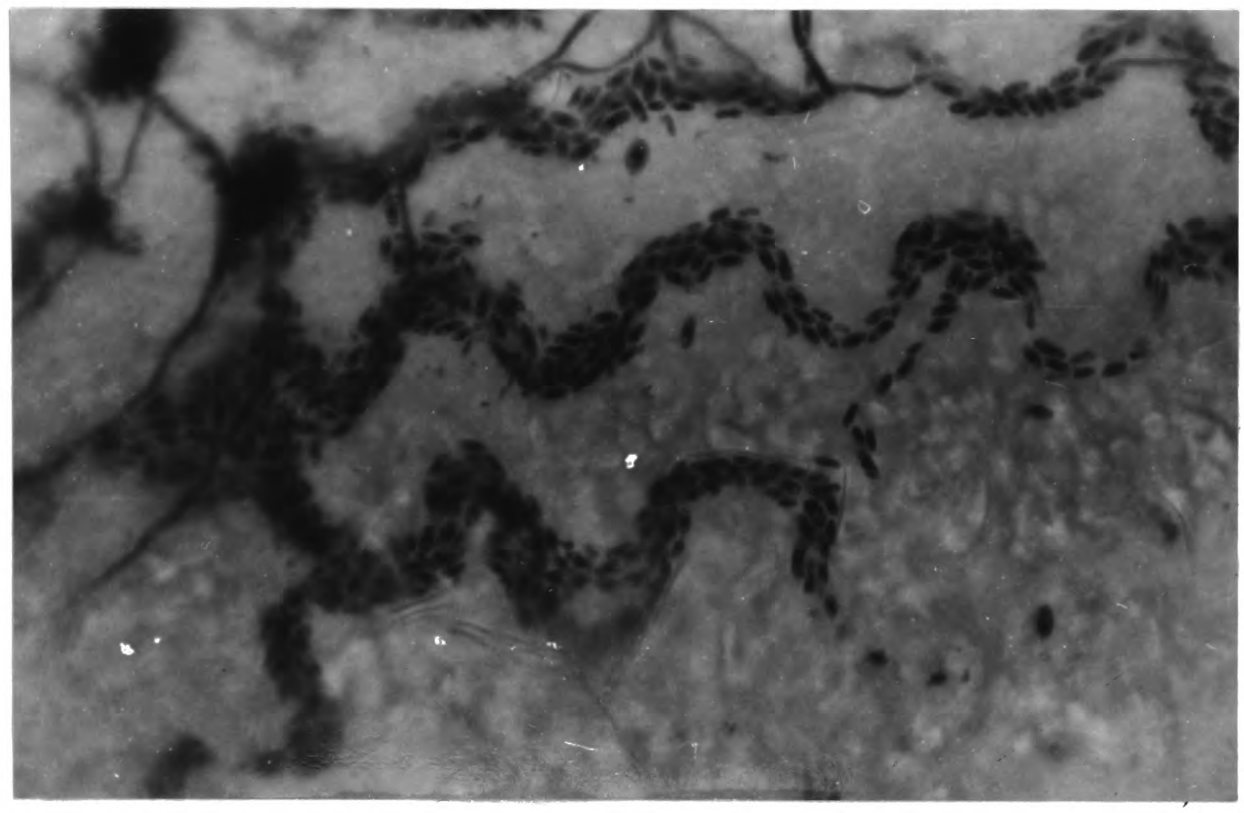
Fig. 27 C. cladosporioides: Germ tube growth on attached leaves of A. Nanum and A. fi hybrid at different times after inoculation

to merge the results from the three replicate experiments on each age of leaf from the two cultivars of antirrhinum, owing to slight differences in the inoculum level achieved on the leaf and the variability between microbial cultures used at different times. Although all the replicates showed similar patterns of colonisation, the difference between replicates might be sufficient to mask any small but important effects occurring. Unless otherwise stated the data presented here are for one experiment and the data for both this and the other experiments are given in Appendix 1.

1) S. ROSEUS

In all experiments the total volume of cells (μm^3) of S. roseus increased over that of the initial inoculum (Fig. 28). There was little difference in the increase in the total volume of cells for the first four days on those leaves bearing cultures of S. roseus alone. From this time onward the volume of cells on older leaves rose dramatically, but at the 14 and 21 day samples the volume of cells on A. Nanum leaves was considerably greater than that on A. Fi hybrid leaves. In these samples aggregations of cells were seen above the anticlinal walls of the epidermal cells of the leaf (Plate 14). The overall increase in volume of cells in 21 days was x 277 on A. Nanum and x 189 on A. Fi hybrid. During the same period the total volume of yeast cells on the younger leaves of both cultivars increased only 30 times. The effect of the presence of C. cladosporioides was marked in that little increase in total volume of cells occurred in the first seven days on all leaves bearing the mixed inoculum. Thereafter, the increase on older leaves, especially A. Nanum, was greater than in younger leaves.

Plate 14. Concentration of S. roseus cells along anticlinal walls of epidermal cells of detached A. Nanum leaf after 14 days incubation (x 395)



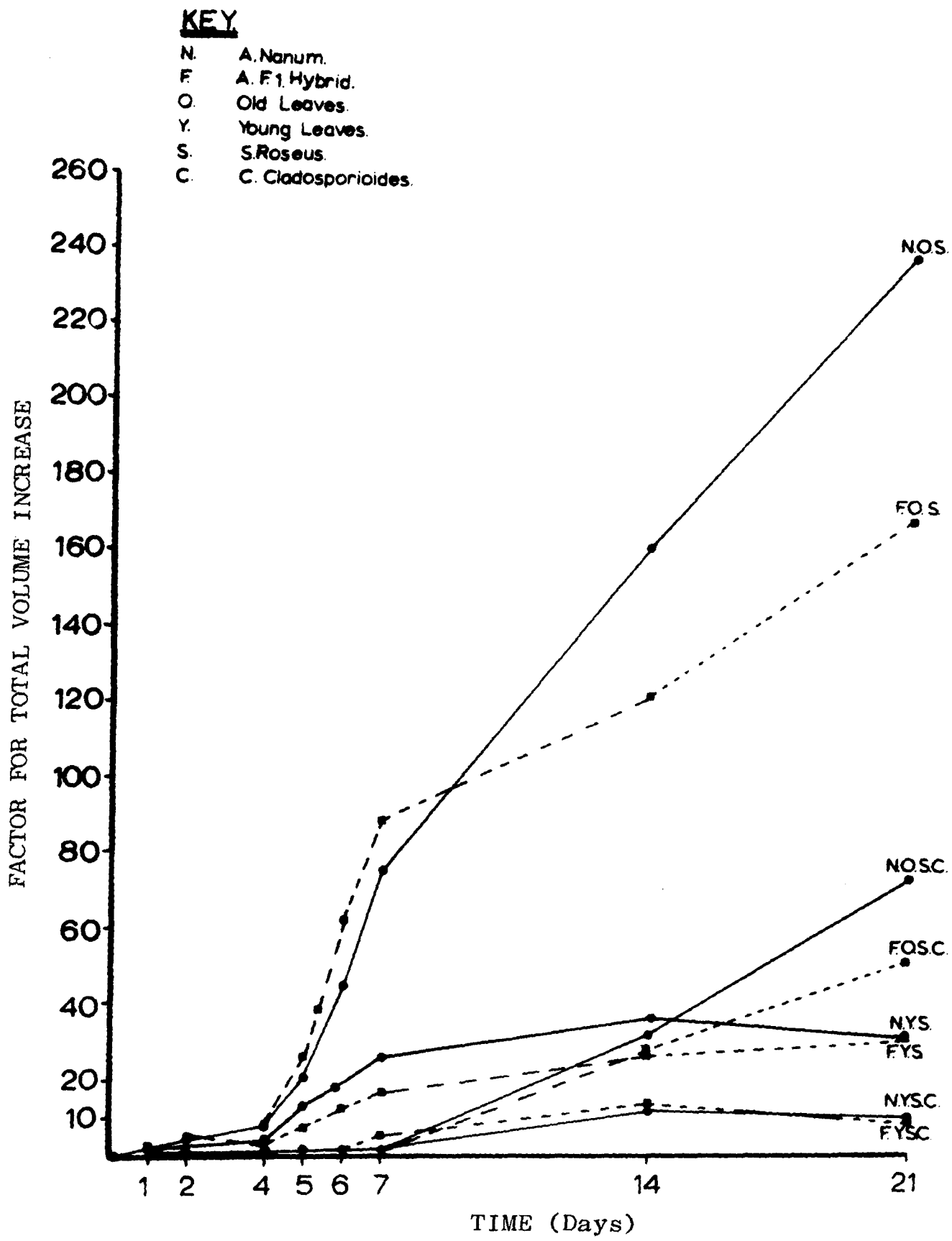


Fig. 28 S. roseus: Increase in total volume of cells cm^{-2} with time on detached leaves of A. Nanum and A. Fi hybrid

The results of the analysis of this data are given as a series of figures showing the differences between means. The range of these differences was so great that the figures had to be reduced by 10^6 and a logarithmic scale applied on two vectors 180^o apart, thus ensuring that positive logarithmic values were used on either side of a reference line. The 95% confidence limits for the difference between the means was obtained using standard techniques and the special procedures necessary for the comparison of means having unequal variances (Bailey, 1968).

The presence of C. cladosporioides led to a significantly lower ($p = .05$) volume of cells of S. roseus on older leaves of both antirrhinum cultivars (except for the day six sample on A. Nanum) compared to the volume when S. roseus alone was incubated on the leaves (Fig. 29). In a similar comparison for the younger leaves the volume of cells of S. roseus was reduced significantly on A. Fi hybrid on days two, four and six and on A. Nanum leaves from day six onwards.

The volume of cells of S. roseus was significantly larger from day four onwards on A. Fi hybrid leaves and day seven onwards on A. Nanum leaves when this yeast was incubated alone (Fig. 30). The results for the mixed cultures with C. cladosporioides were similar for both cultivars of antirrhinum. From the day one sample onward on A. Nanum, and the day two sample onward on A. Fi hybrid, the mean values for the total volume of cells were higher on the younger leaves, but the difference between means was significant only on day six for A. Nanum and on day five for A. Fi hybrid. After day seven the older leaves supported a larger volume of cells which was significantly higher ($p = .05$) on day twenty-one for A. Nanum and samples on days fourteen and twenty-one on A. Fi hybrid leaves.

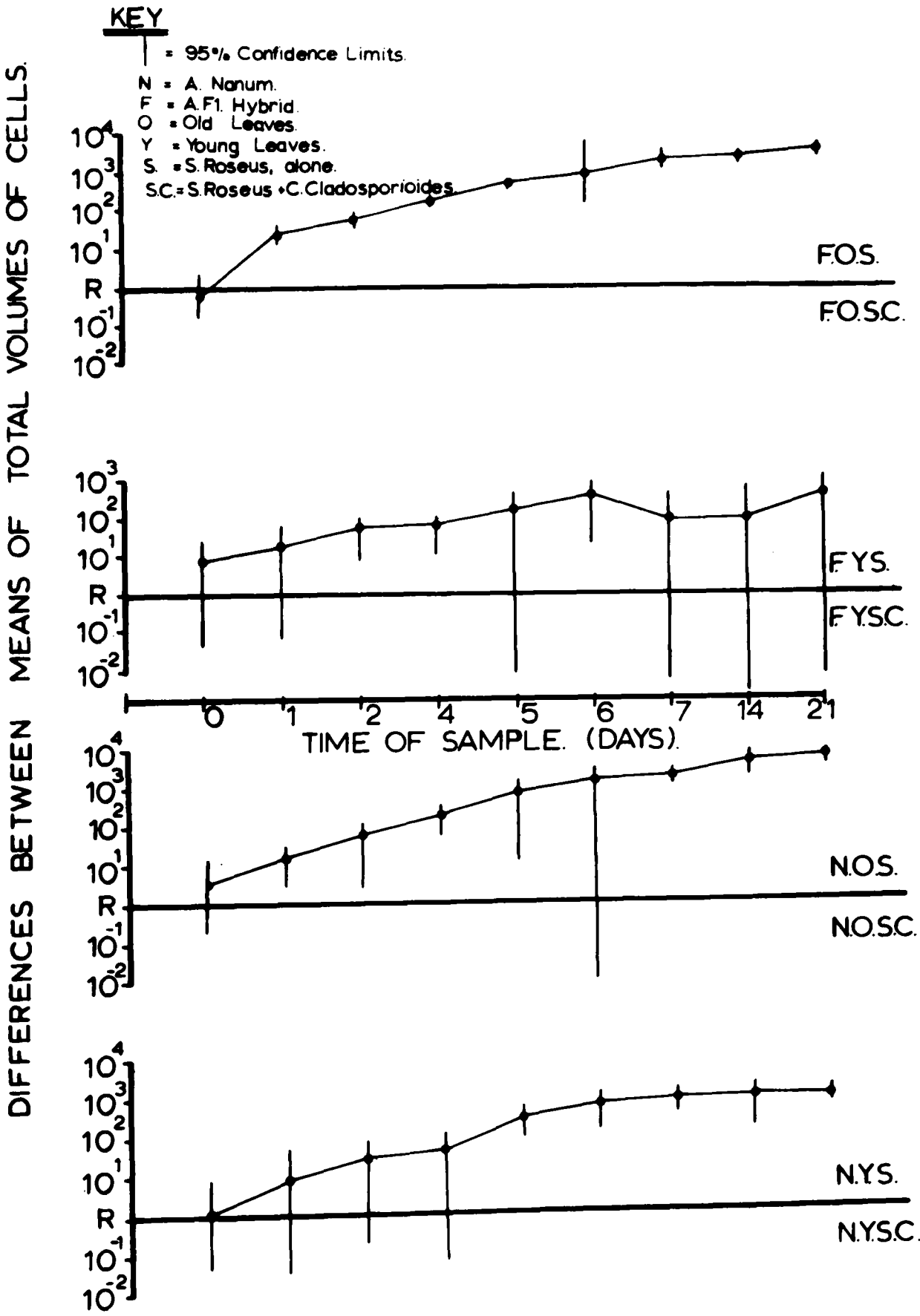


Fig. 29 S. roseus: Effect of the presence of C. cladosporioides on the total volume of cells $cm^{-2} \times 10^{6}$ produced on detached antirrhinum leaves

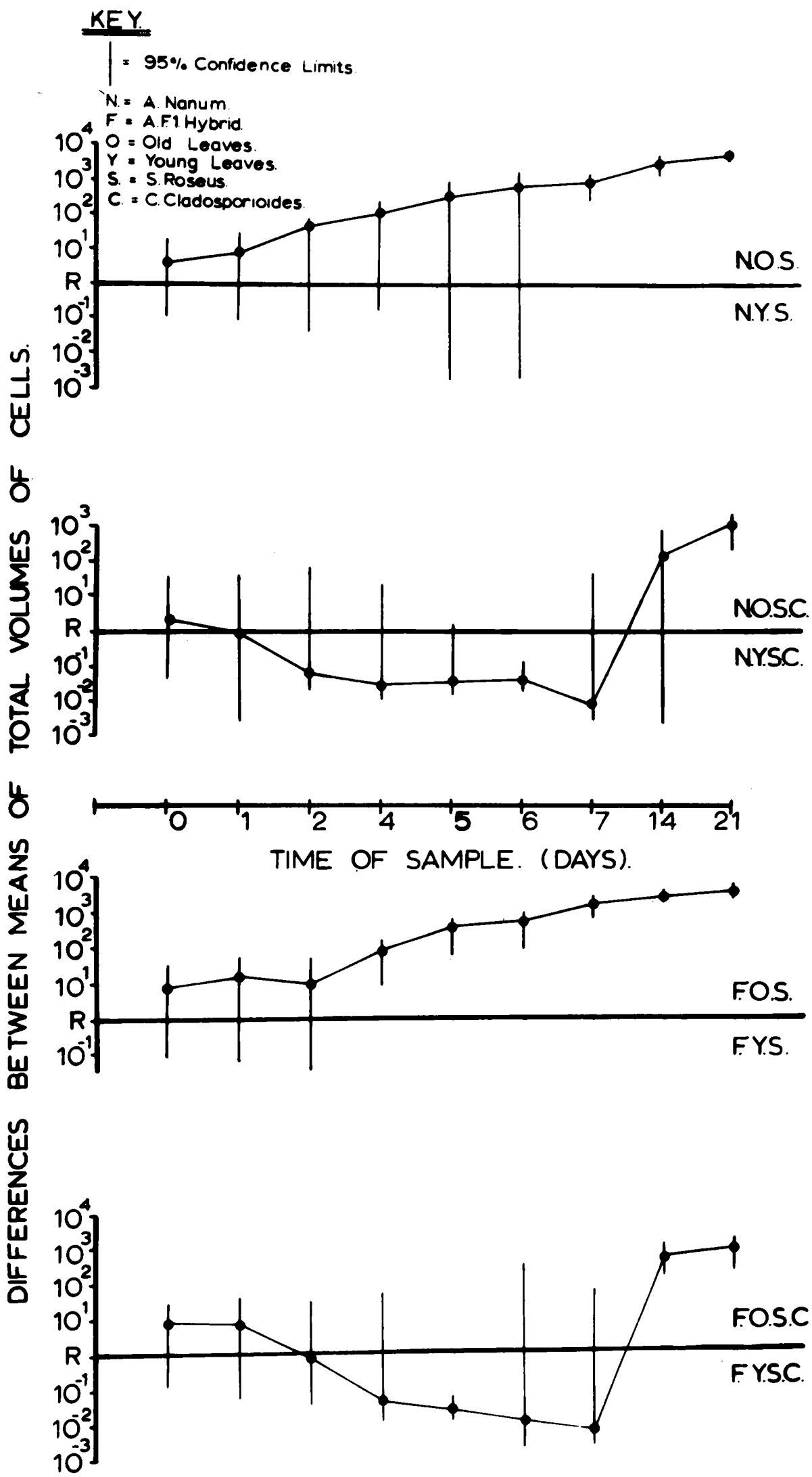


Fig. 30 S. roseus: Effect of leaf age on the total volume of cells $cm^{-2} \times 10^6$ produced on detached antirrhinum leaves

Older A. Nanum leaves supported a significantly greater ($p = .05$) volume of cells of S. roseus in the twenty-one day sample (Fig. 31). For older leaves inoculated with S. roseus plus C. cladosporioides, A. Nanum supported a significantly greater volume of cells on the day six sample only. Otherwise there was little difference between the cultivars. Comparison between younger leaves of each cultivar inoculated with S. roseus alone showed a significant difference ($p = .05$) between the mean total volume of cells on day six only. Similarly for the mixed inoculum, the means were only significantly different in samples on day seven and fourteen. Thus, between the young leaves of each cultivar no clear cut difference could be discerned.

In addition the changes in the population of S. roseus on leaf surfaces were monitored using the sporefall technique. The results were treated using a logarithmic transform (Kleczkowski, 1949) which had been used successfully for the results of this isolation procedure in a previous study (Last, 1955a). Using this technique it was not possible to isolate S. roseus prior to the fourteen day sample in any experiment (Table 26). On the fourteenth day S. roseus was isolated from the older leaves of both cultivars of antirrhinum which had been inoculated with this yeast alone. At the twenty-one day sample S. roseus was isolated from all treatments on both young and old leaves. The numbers of S. roseus isolated from leaves inoculated with the yeast alone were higher than from those leaves inoculated with mixed inoculum. In addition, higher numbers were obtained from older leaves compared with younger leaves. However, there seemed to be little difference between leaves of the same age of the two cultivars of antirrhinum subjected to the same inoculum.

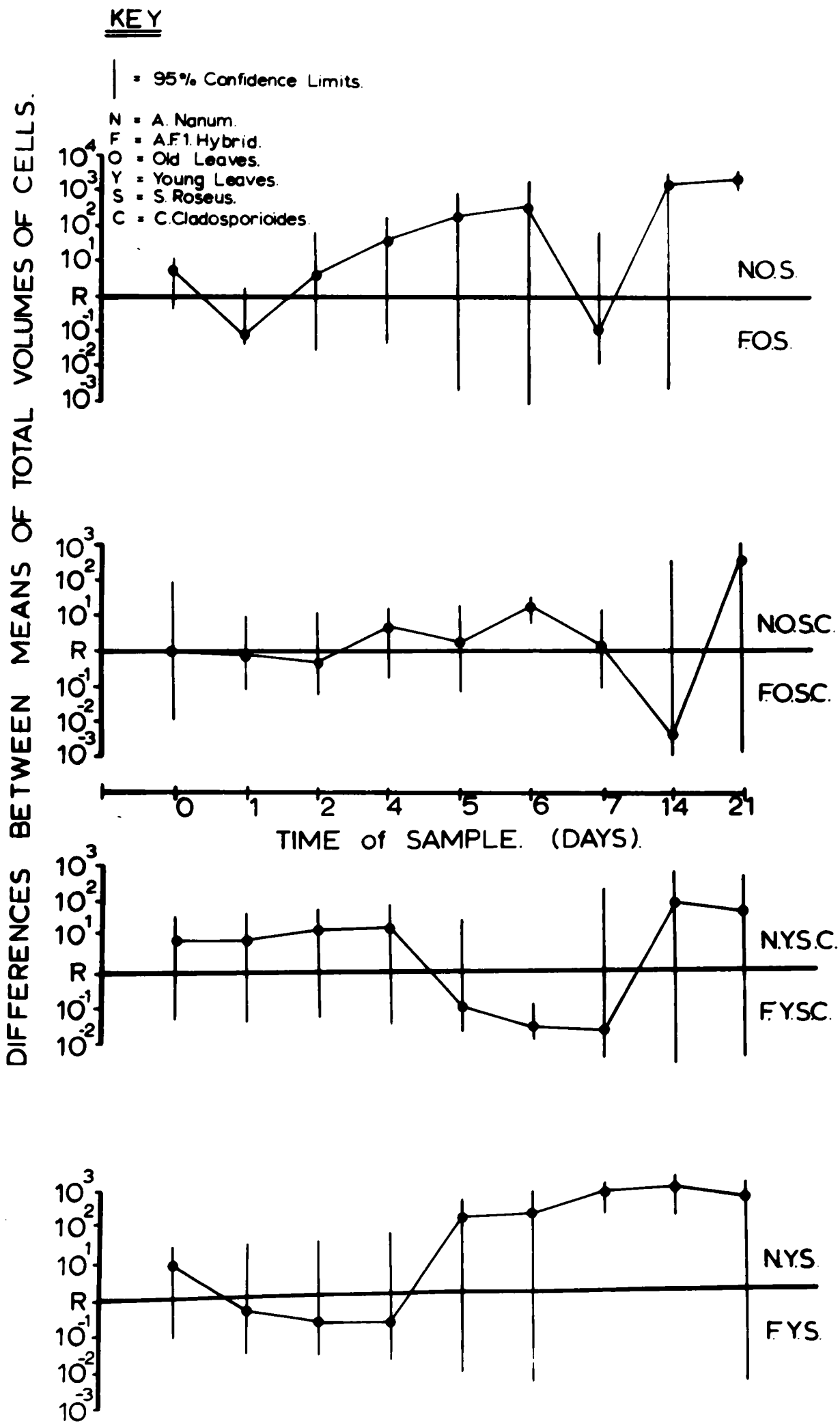


Fig. 31 S. roseus: Effect of cultivar of antirrhinum on the total volume of cells $cm^{-2} \times 10^6$ produced on detached antirrhinum leaves

Table 26 S. roseus: Numbers of colonies (cm^{-2}) isolated by sporefall method from detached leaves of A. Nanum and A. Fi hybrid at different times after inoculating the leaves with S. roseus or S. roseus plus C. cladosporioides

Cultivar	Leaf age	Treatment	Numbers cm^{-2} Incubation (Days) 14	21
A. Nanum	older	S	2.5	14.2
		S + C	0	4.0
	younger	S	0	4.9
		S + C	0	0.5
A. Fi hybrid	older	S	2.7	15.6
		S + C	0	3.0
	younger	S	0	4.8
		S + C	0	0.8

2) C. CLADOSPORIOIDES

In all experiments the percentage germination of spores and germ tube lengths increased from the initial zero value. The figures of the data presented here do not depict this initial value in order not to confuse the values of observations taken during the early period after inoculation.

The data for the percentage spore germinationwere transformed using the arc sin transform (Bliss, 1937), tables and procedures for which are readily available (Snedecor & Cochrane, 1967) to enable statistics based on a normal distribution to be used. When the older leaves of A. Nanum were inoculated with C. cladosporioides alone, after one day's incubation the percentage germination on the leaf midrib was significantly higher ($p = .05$) than on the leaf lamina (Fig. 32). After a further increase on the leaf midrib the percentage germination decreased after four days, but on the leaf lamina it rose to a peak on the fifth day before declining. Both the initial increases in percentage germination and the subsequent decreases to the twenty-one day sample were significant ($p = .05$). When S. roseus was present in the inoculum these decreases were delayed for two days. It should be noted that prior to the six day sample the leaves inoculated with C. cladosporioides alone showed a higher mean percentage germination than leaves inoculated with the mixed C. cladosporioides, S. roseus culture. The converse was true after six days. In addition, after the four day sample secondary spore formation was observed (Plate 15).

On younger leaves the pattern of spore germination was different, as after the initial rapid rise in germination from zero to 29.6% within one day, the germination percentage remained almost the same

Plate 15. Conidiophore and conidia formation in C. cladosporioides after five days incubation on detached leaf (lamina) of A. Nanum (x 1240)

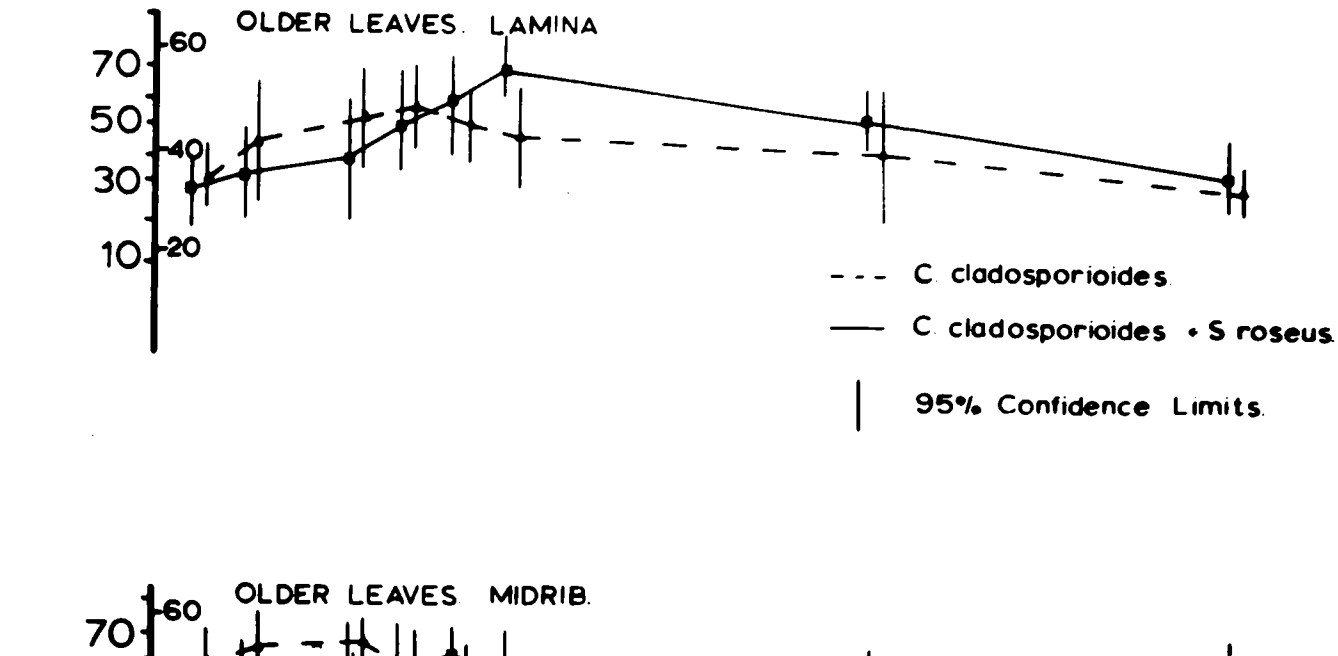


Fig. 32 C. cladosporioides: Spore germination on detached leaves of A. Nanum at different times after inoculation

throughout the twenty-one day period of the experiment. No difference in germination could be discerned between the leaf midrib and lamina. The mean values of spore germination on leaves inoculated with C. cladosporioides alone were consistently higher than those from leaves inoculated with C. cladosporioides plus S. roseus although the confidence limits crossed for each pair of observations.

Data from observations on leaves of A. Fi hybrid (Fig. 33) was similar to that described above for the leaves of A. Nanum. However, on older leaves inoculated with C. cladosporioides alone the percentage germination on the leaf midrib was significantly higher ($p = .05$) on days one, two and four than on the leaf lamina. The peak in germination occurred in five days on both areas of the leaf for C. cladosporioides incubated alone, and at six days in mixed culture treatments. The cultures on the younger leaves showed the same overall pattern as those observed on A. Nanum, of an initial rise in the first day and then little change over the remainder of the experiment. Again the germination of C. cladosporioides alone was consistently higher than in those cultures where S. roseus was also present.

The spore germ tube length data were difficult to analyse. The distribution of germ tube lengths within a sample was not normal and wide variation was observed in individual germ tube lengths on any one sampling occasion. The shape of this distribution changed with time. As insufficient data were available to allow the development of a transformation to normalise the data, it was decided, on statistical advice from Mr. E. Renshaw of Edinburgh University Department of Statistics, that the best way to handle the

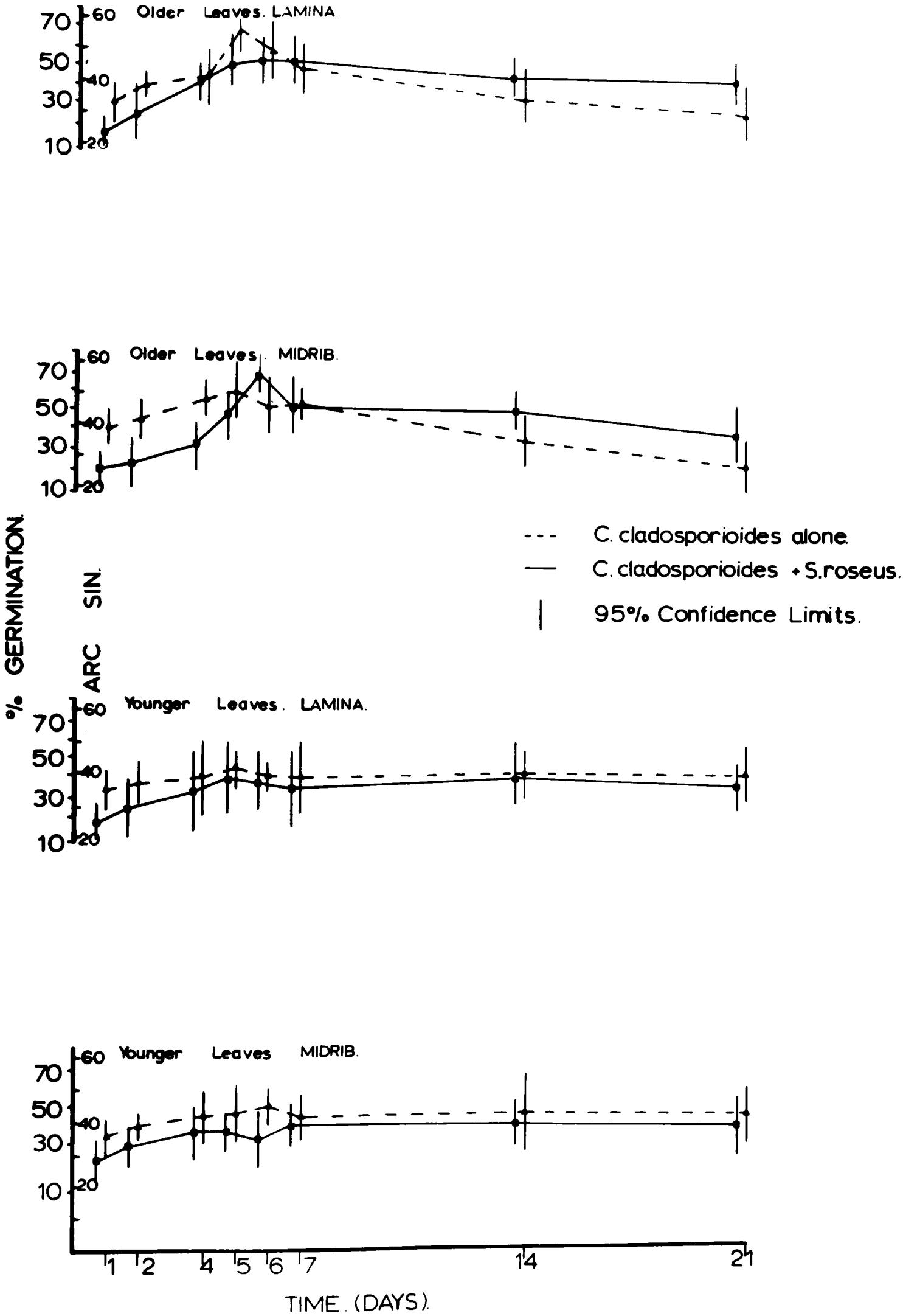


Fig. 33 C. cladosporioides: Spore germination on detached leaves of A. Fi hybrid at different times after inoculation

accumulated data was to present it in the form of mean values for each sampling period and display the variability within the data by illustrating one standard deviation about the mean. It should be emphasised that no statistical significance should be attached to the value of the standard deviation.

The data for germ tube length of C. cladosporioides on the leaves of A. Nanum (Fig. 34) shows that after an initial rapid increase in length within the first day of incubation (Plate 16) on both younger and older leaves the rate of germ tube growth slowed (Plate 17) on both leaf lamina and midrib. After seven days the germ tube lengths increased markedly until the end of the experiment, from 22–23 µm to 40.9–43 µm on younger leaves and from 32–37 µm to 227–243 µm on older leaves. These figures were for C. cladosporioides alone, but similar increases were observed on leaves inoculated with mixed cultures. Little difference in germ tube lengths was noted in samples from the lamina and midrib areas of the leaves. This was true for samples from the same age of leaf throughout the twenty-one days of the experiment.

A similar pattern of results was obtained for germ tube lengths of C. cladosporioides on leaves of A. Fi hybrid (Fig. 35). After the initial growth to about 10–20 µm within one day there was little change in mean germ tube length for another 5–6 days after which time the mean germ tube lengths started to increase again. This increase was from 20–25 µm to 43 µm on younger leaves and from 23–29 µm to 81–108 µm on older leaves. Again, there was little difference between the observations from the leaf lamina compared to those from the leaf midrib.

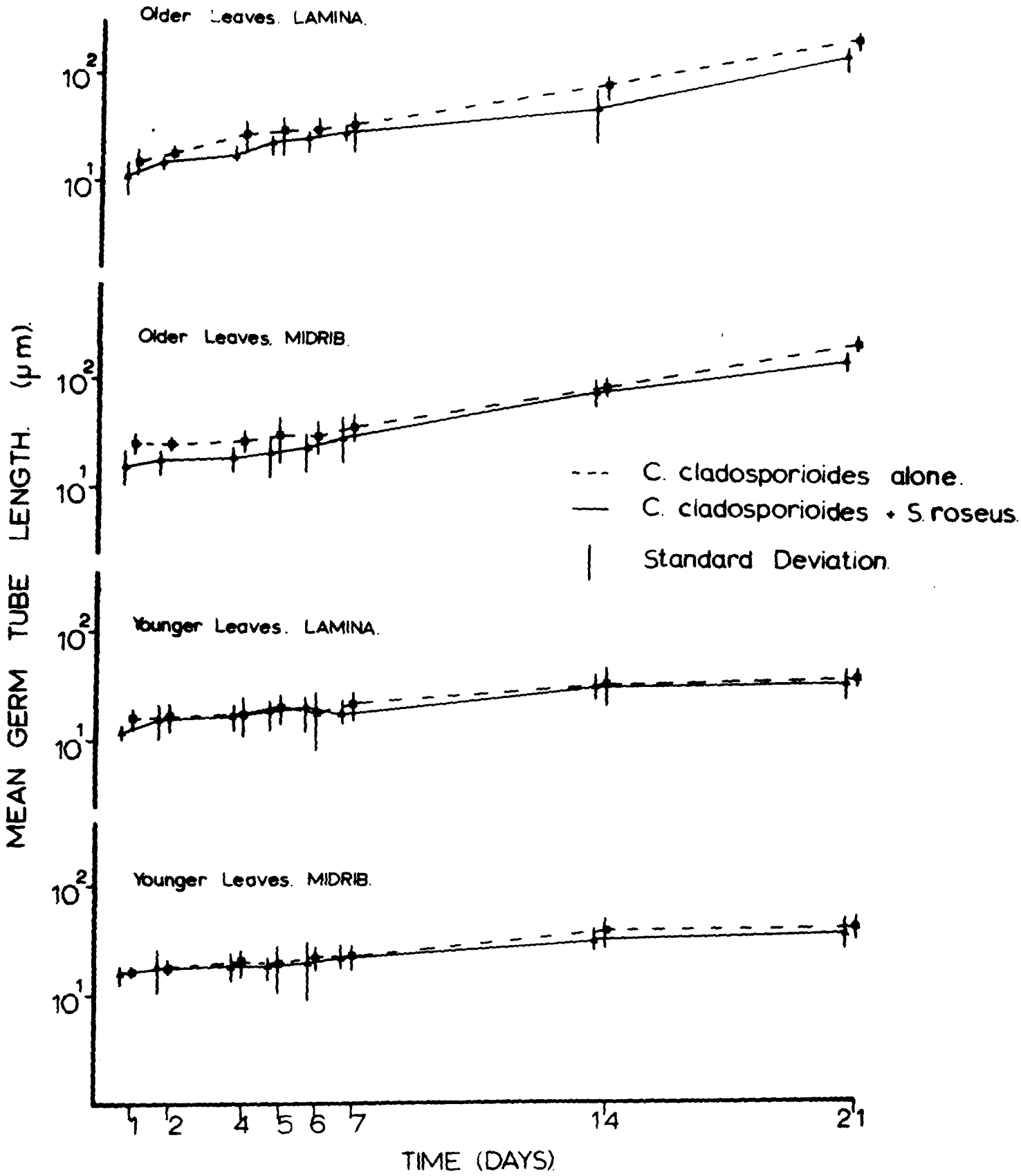


Fig. 34 C. cladosporioides: Spore germ tube growth on detached leaves of A. Nanum at different times after inoculation

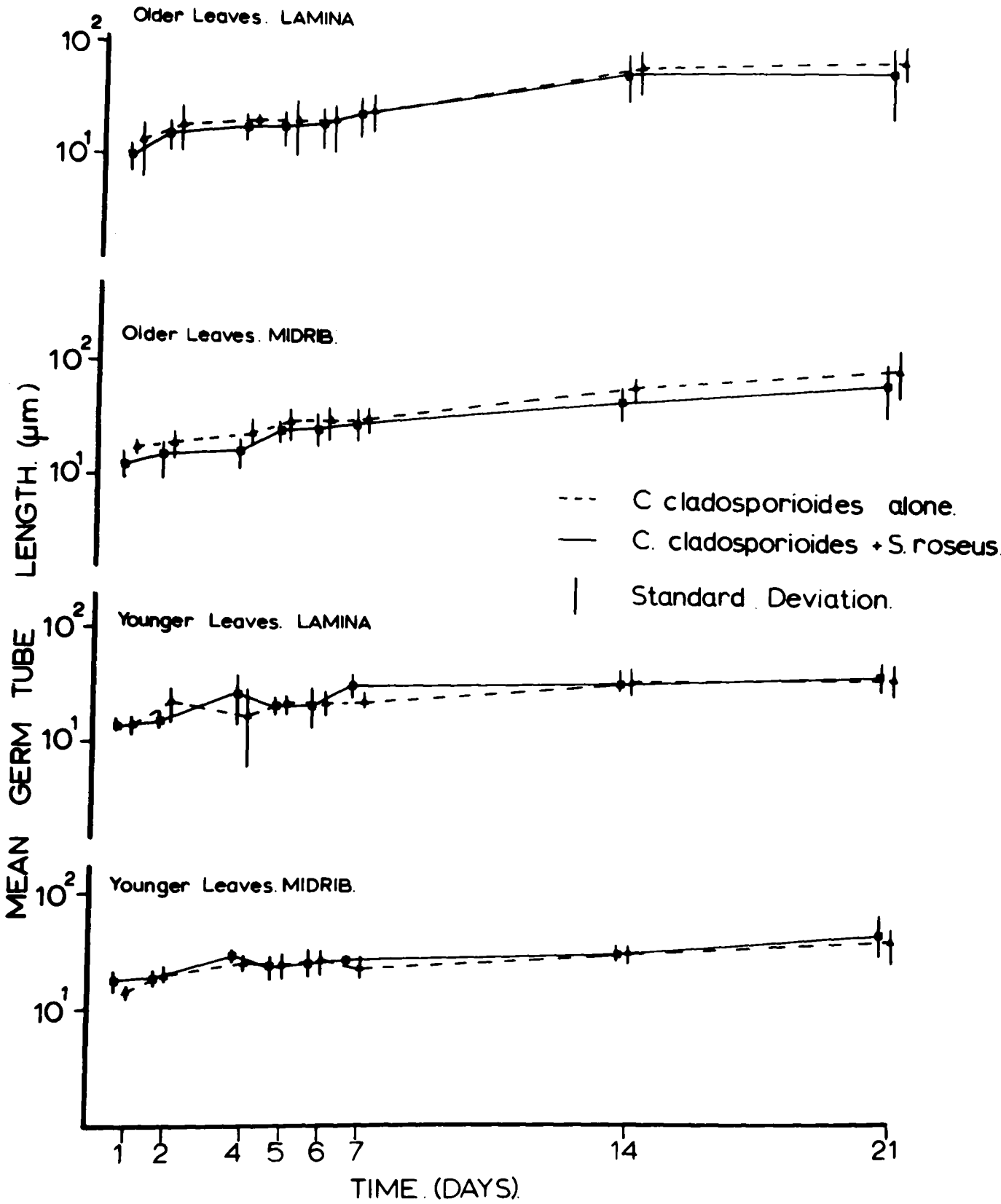


Fig. 35 C. cladosporioides: Spore germ tube growth on detached leaves of A. Fi hybrid at different times after inoculation

Plate 16 Germination of C. cladosporioides spores on detached leaf of A. Nanum after one day's incubation (x 2270)

Plate 17. Germination of C. cladosporioides spores on detached leaf of A. Fi hybrid after two days incubation (x 1730)

1) EXPERIMENTS IN VITRO

a) P. antirrhini

The observations on P. antirrhini (Fig. 36) showed that the germination of uredospores varied appreciably, but not significantly ($p = .05$) when these spores were incubated with saprophytic micro-organisms. The median germ tube lengths are also given in this figure, together with a matrix showing observed differences which were significant ($p = .05$). When P. antirrhini was incubated with S. roseus, the germ tube length of P. antirrhini was reduced relative to the control (P. antirrhini alone), but increased when incubated with both C. cladosporioides alone and with C. cladosporioides in combination with S. roseus.

b) C. cladosporioides

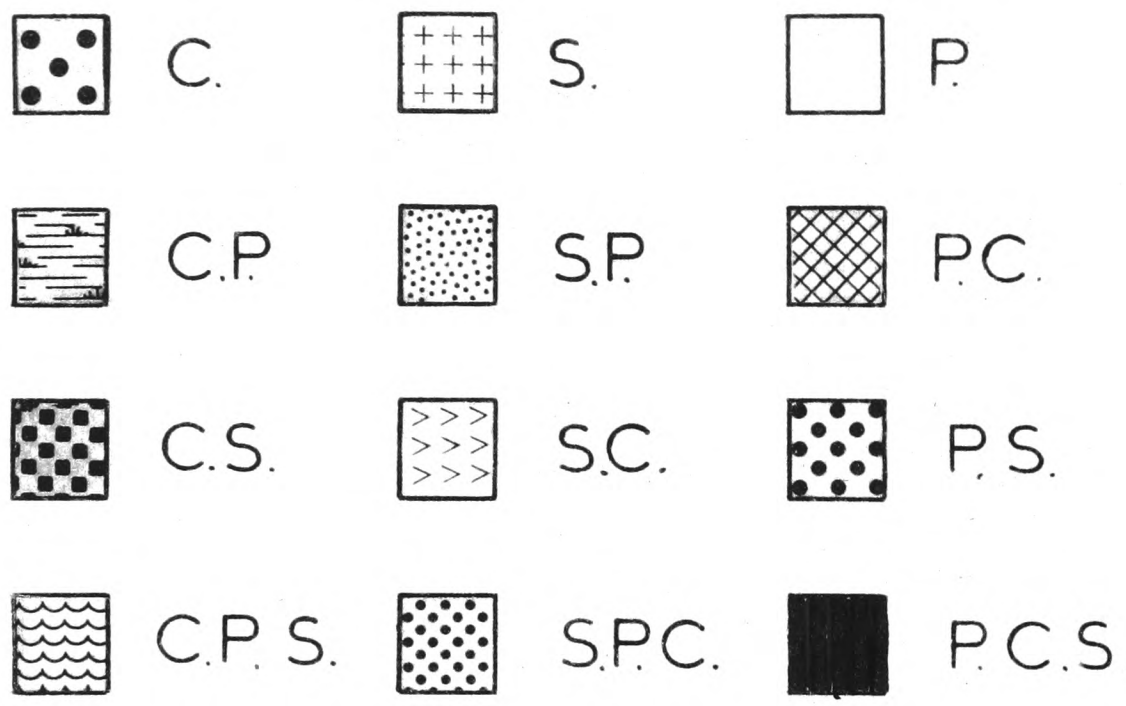
The observations for C. cladosporioides (Fig. 37) show that spore germination was reduced in the presence of the other two organisms. However, this reduction was only significant ($p = .05$) when the conidia of C. cladosporioides were incubated with P. antirrhini alone. The data on median germ tube length also show reductions in the presence of the other organisms, relative to the control, but in these cases the majority of the observed values were significantly different ($p = .05$), as shown in the matrix (Fig. 37).

c) S. roseus

The total volume of cells of S. roseus was significantly greater ($p = .05$) when incubated with the other micro-organisms than when incubated alone (Fig. 38). The largest increase was observed when the yeast was incubated with P. antirrhini. The addition of C. cladosporioides to this mixture reduced the total

Key for Figures 36-48

Microbes present in inocula



P = P. antirrhini alone

PC = P. antirrhini + C. cladosporioides

PS = P. antirrhini + S. roseus

PCS = P. antirrhini + C. cladosporioides + S. roseus

C = C. cladosporioides alone

CP = C. cladosporioides + P. antirrhini

CS = C. cladosporioides + S. roseus

CPS = C. cladosporioides + P. antirrhini + S. roseus

S = S. roseus alone

SP = S. roseus + P. antirrhini

SC = S. roseus + C. cladosporioides

SPC = S. roseus + P. antirrhini + C. cladosporioides

Statistics

Germination and leaf penetration data: 95% Confidence limits on transformed data (arc sin trnasform) represented as a vertical bar.

Germ tube length data: Significant differenees ($p = .05$) as calculated using the Mann Whitney 'U' test, given in the matrices under the relevant figures.

Total volume of yeast cells: 95% Confidence limits of the mean from raw data are represented as a vertical bar.

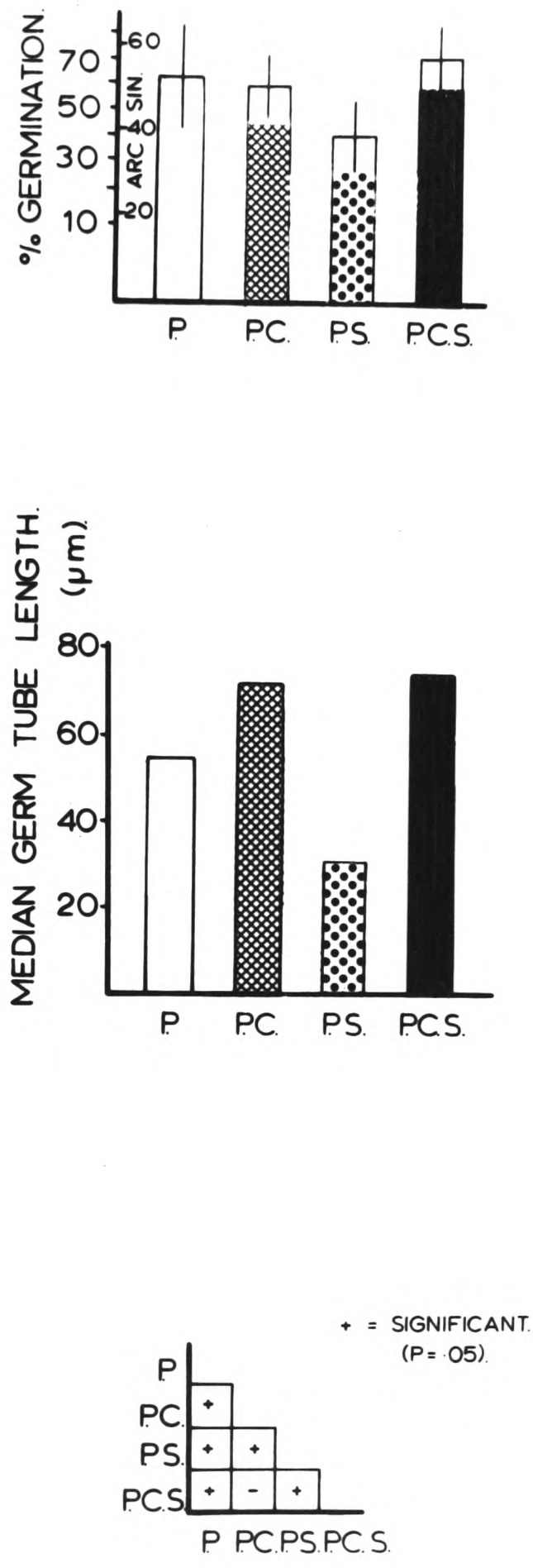
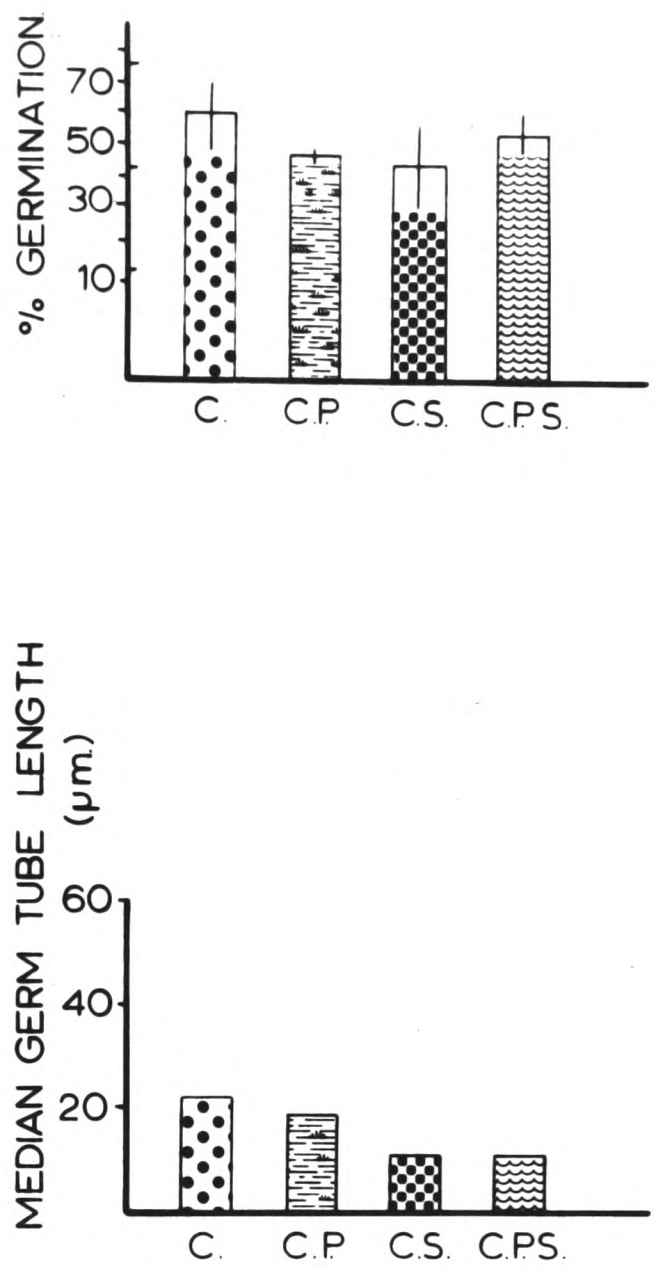


Fig. 36 P. antirrhini: Interactions in vitro with single and mixed cultures of saprophytic phylloplane organisms: Germination and germ tube growth



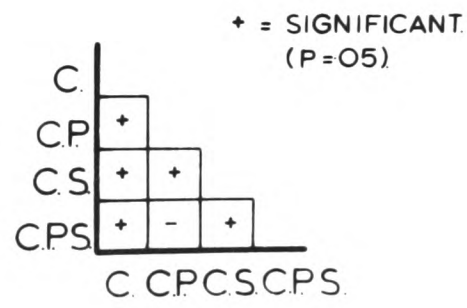


Fig. 37 C. cladosporioides: Interactions in vitro with single and mixed cultures of micro-organisms: Germination and germ tube growth

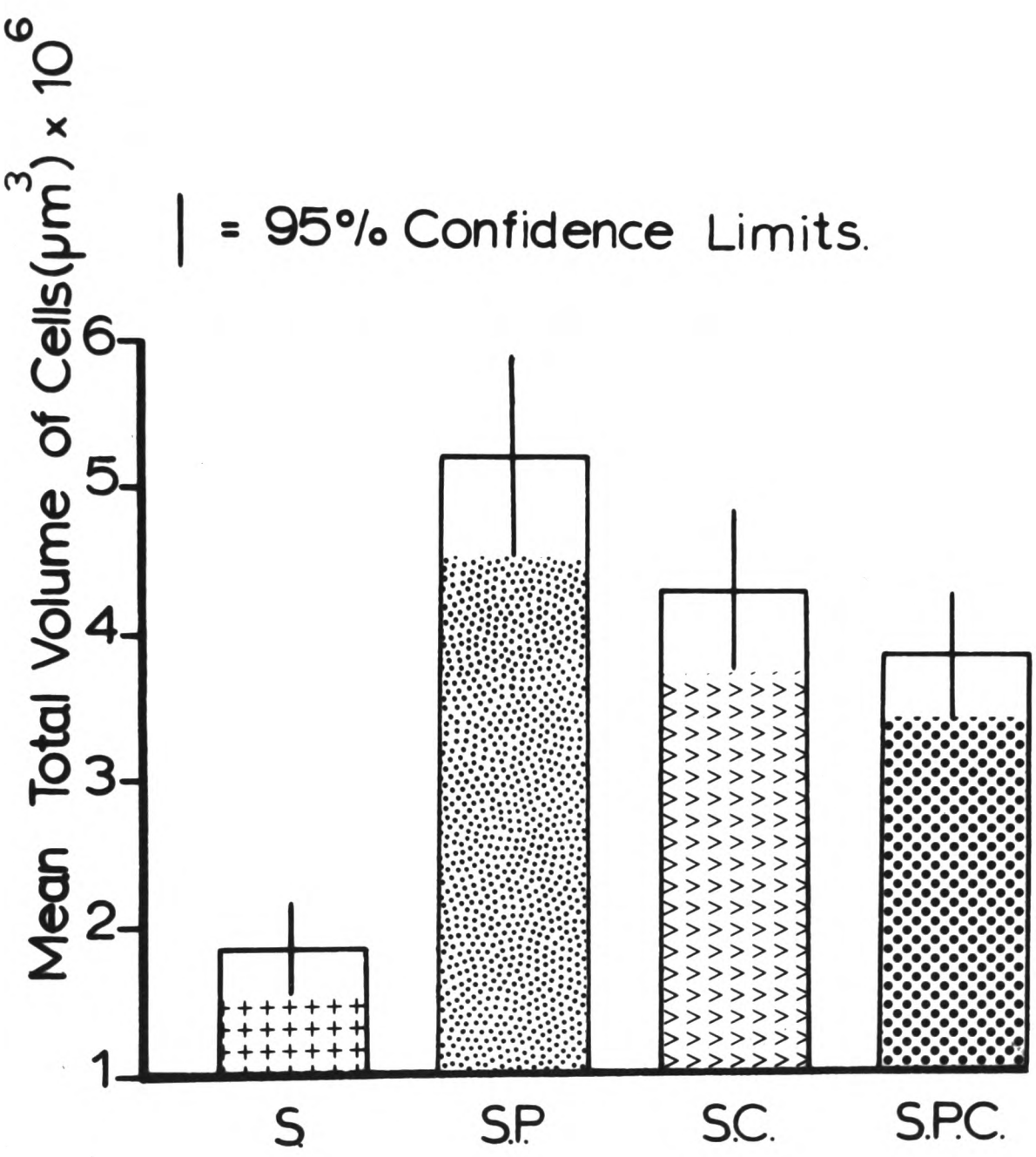


Fig. 38 S. roseus: Interactions in vitro with single and mixed cultures of micro-organisms: Mean total volume of cells (μm^3)

volume of cells significantly ($p = .05$), and S. roseus incubated with C. cladosporioides alone gave an intermediate value.

INTERACTIONS ON ROOTED DETACHED LEAVES

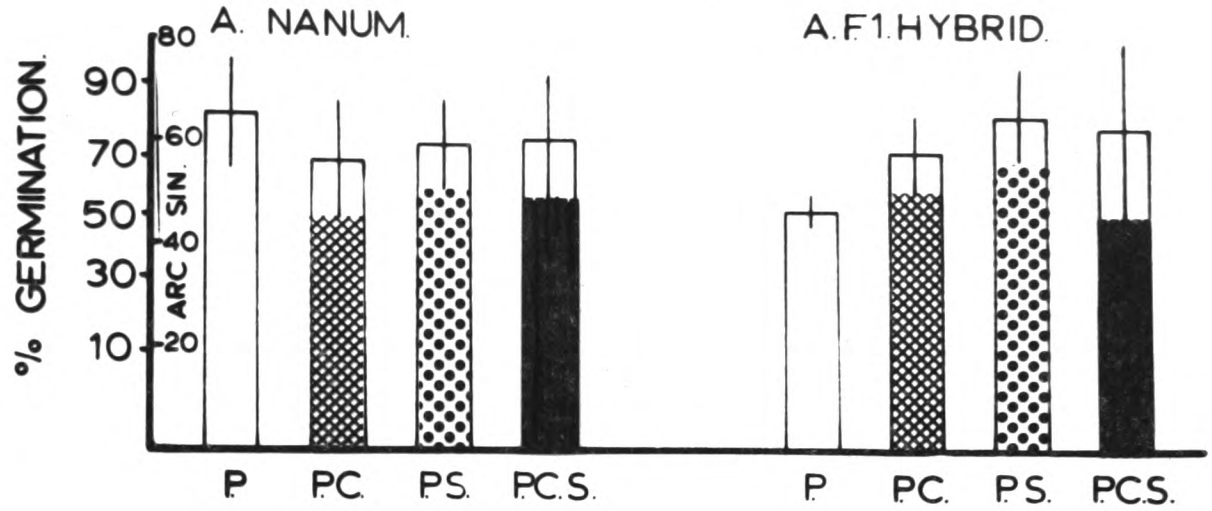
The observations of the two different environmental regimes employed will be considered separately. The results given here refer to one experiment only. The similar results of the second experiment on detached leaves are contained in Appendix 1.

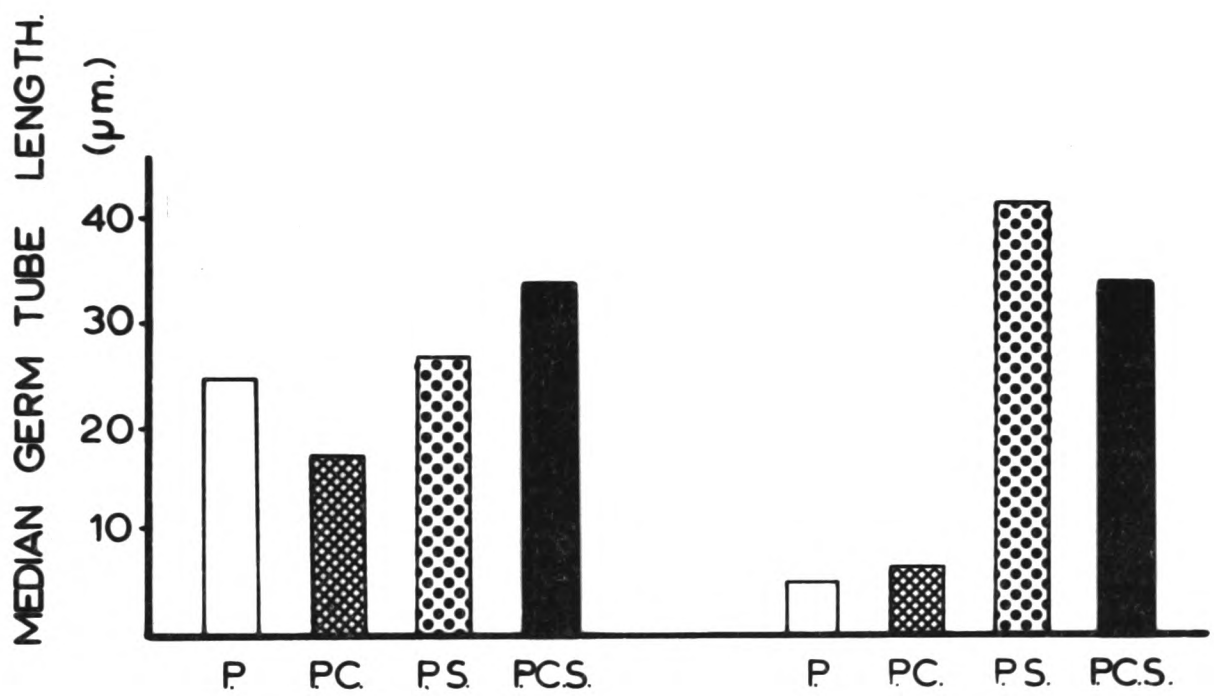
a) Two days incubation (10°C; darkness)

i) P. antirrhini

The mean values for uredospore germination on A. Nanum leaves (Fig. 39) in each combination of micro-organisms showed a decrease (not significant) relative to the control. However, on A. Fi hybrid the mean percentage germination was higher in the presence of either of the saprophytic micro-organisms. This difference was significant ($p = .05$) when P. antirrhini was incubated with either C. cladosporioides or S. roseus alone, but not when all three organisms were incubated together. The 52% germination achieved by P. antirrhini alone on leaves of A. fi hybrid was significantly ($p = .05$) lower than the 81% achieved on leaves of A. Nanum.

The presence of S. roseus alone or in combination with C. cladosporioides led to significantly larger median germ tube lengths on both cultivars of antirrhinum (Fig. 39). The presence of C. cladosporioides alone did not affect the median length of germ tubes relative to the control. On A. Nanum leaves the greatest increase was recorded whenboth saprophytes were present, but on A. Fi hybrid the greatest increase was recorded when S. roseus alone





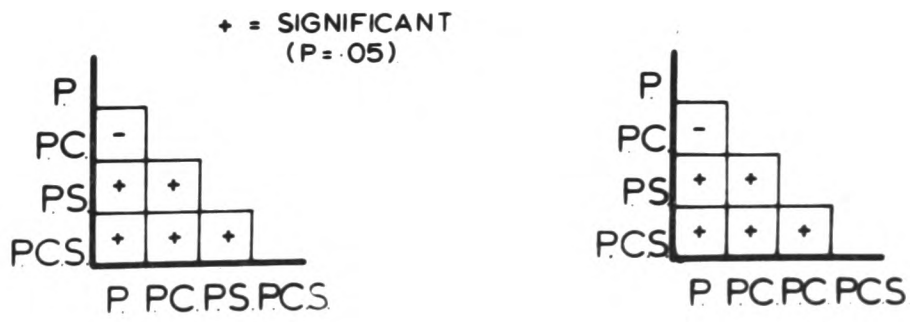


Fig. 39 P. antirrhini: Interactions on detached leaves with single and mixed cultures of saprophytic phylloplane micro-organisms: Spore germination and germ tube growth (2 day test)

was incubated with P. antirrhini.

A comparison of the germ tube lengths of P. antirrhini produced by equivalent treatments (mixtures of micro-organisms) on the leaves of A. Nanum and A. fi hybrid (Table 27) shows, that for P. antirrhini alone, or incubated with C. cladosporioides, the germ tube lengths recorded on A. Fi hybrid were significantly lower ($p = .05$) than those on A. Nanum. In contrast, when P. antirrhini was incubated with S. roseus the recorded germ tube lengths on A. Fi hybrid were significantly ($p = .05$) higher than those on A. Nanum.

Data on the penetration of the leaf by P. antirrhini (Fig. 40) show that although the mean percentages were lower in all treatments when saprophytes were present, the difference was in no case significant ($p = .05$) on leaves of A. Nanum. However, on leaves of A. Fi hybrid a significant increase was recorded when P. antirrhini was incubated with S. roseus alone, but not when C. cladosporioides was also present. The reduction in penetration observed in the presence of C. cladosporioides alone was not significant ($p = .05$).

ii) C. cladosporioides

The data for C. cladosporioides was more complex in that the leaf lamina and midrib were assessed separately on each leaf both for spore germination and for germ tube length.

Spore germination of C. cladosporioides on the midrib of A. Nanum leaves was significantly reduced, relative to the control of C. cladosporioides alone, when it was incubated with P. antirrhini and S. roseus together (Fig. 41). In all other treatments the

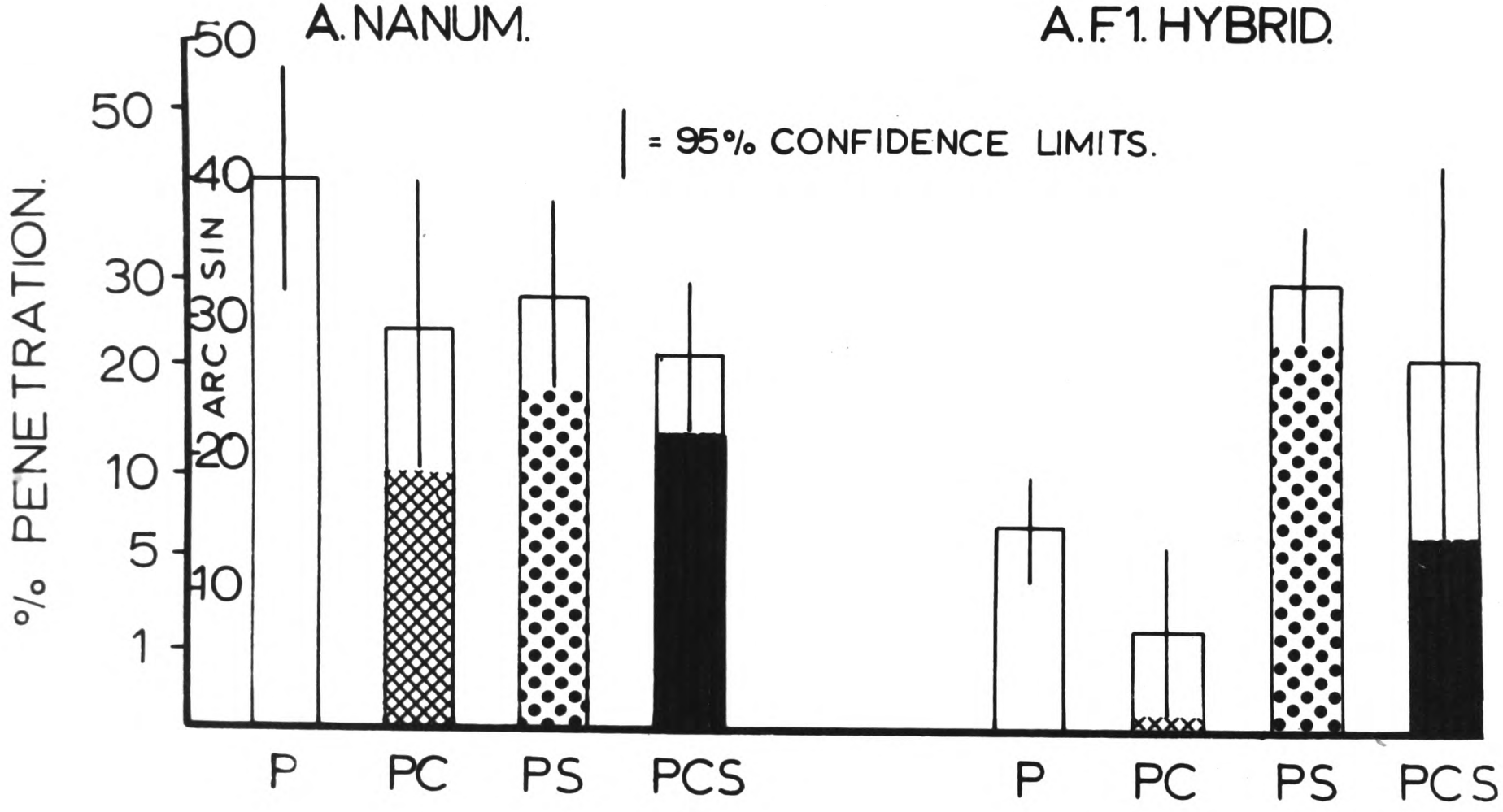
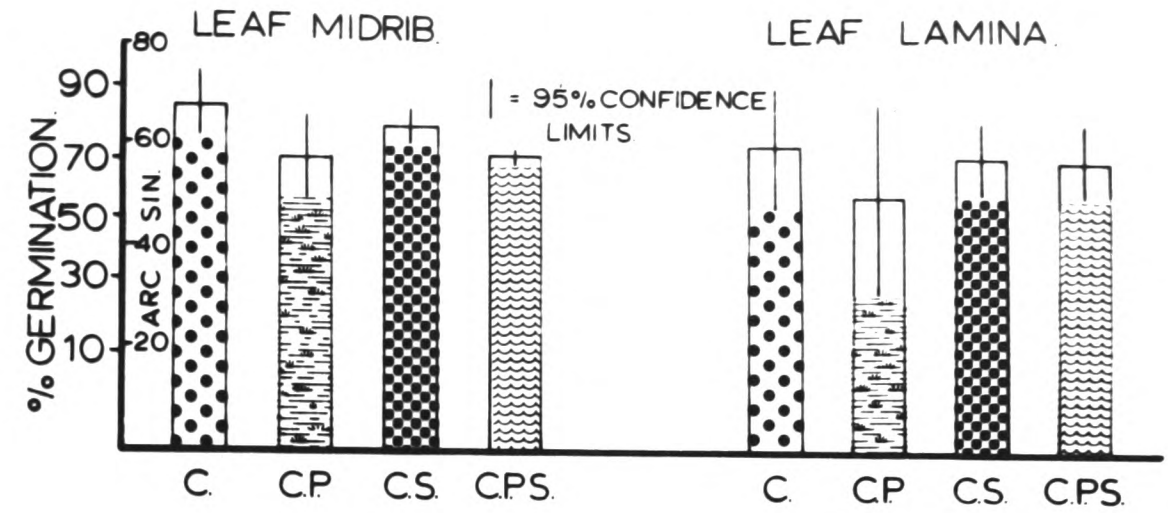
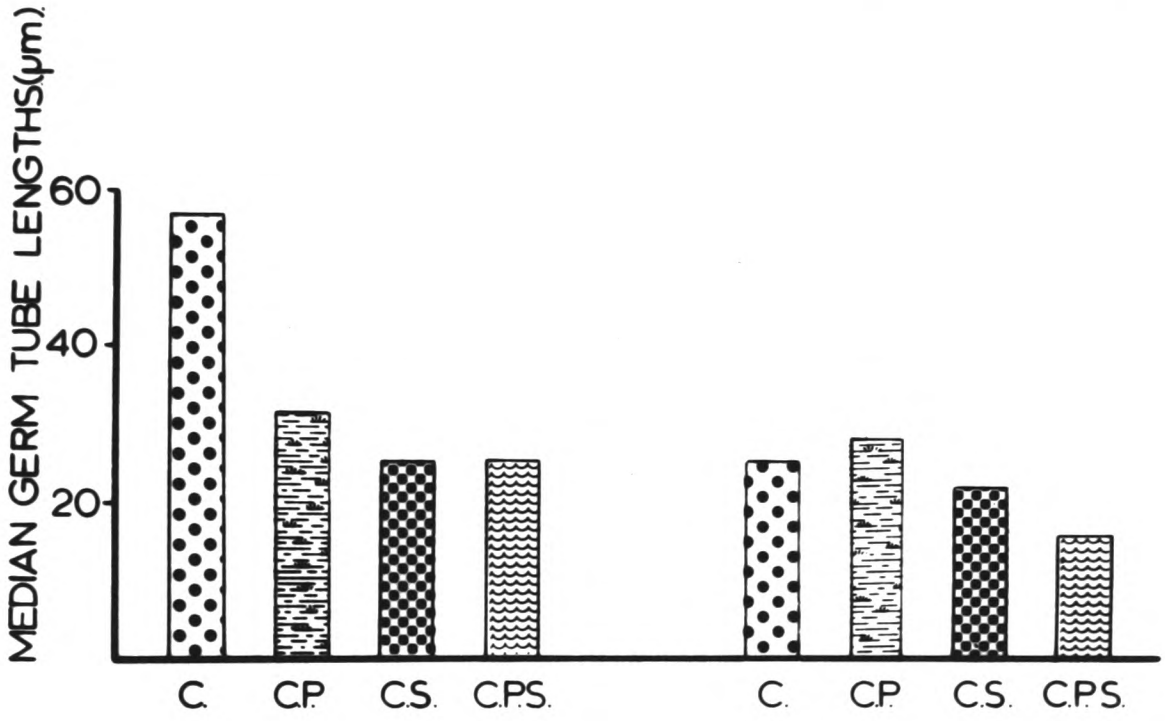


Fig. 40 P. antirrhini: Interactions on detached leaves with single and mixed cultures of saprophytic phylloplane micro-organisms:
Leaf penetration (2 day test)





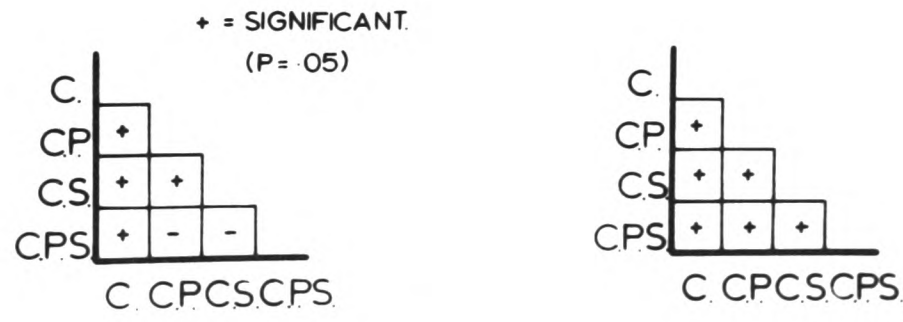


Fig. 41 C. cladosporioides: Interactions on detached leaves of A. Nanum with single and mixed cultures of micro-organisms: Spore germination and germ tube growth (2 day test)

Key for Tables 27-40

Interactions on detached leaves: Tables depicting the statistical differences of the observed parameters

1) Microbial inocula

a) P. antirrhini

P = P. antirrhini alone
PC = P. antirrhini + C. cladosporioides
PS = P. antirrhini + S. roseus
PCS = P. antirrhini + C. cladosporioides + S. roseus

b) C. cladosporioides

C = C. cladosporioides alone
CP = C. cladosporioides + P. antirrhini
CS = C. cladosporioides + S. roseus
CPS = C. cladosporioides + P. antirrhini + S. roseus

c) S. roseus

S = S. roseus alone
SP = S. roseus + P. antirrhini
SC = S. roseus + C. cladosporioides
SPC = S. roseus + P. antirrhini + C. cladosporioides

2) Plant cultivar

N = A. Nanum
F = A. Fi hybrid

3) Location on leaf

M = Midrib
L = Lamina

4) Test

2 = Observation of parameter in 2 day test was greater
6 = Observation of parameter in 6 day test was greater

5) Statistical significance

* = Significant difference (p = .05)

Interactions on detached leaves: Table depicting the statistical differences of the observed parameters (Tables 27-40)

Table 27 P. antirrhini: Germ tube lengths: Comparison between cultivars of antirrhinum for each treatment (2 day test)

Treatment	P	P+C	P+S	P+S+C
Significance	*N	*N	*F	-

Table 28 C. cladosporioides: Germ tube length: Comparison between different locations on the leaf surface (2 day test)

	Cultivar	
Treatment	Nanum	Fi hybrid
C	*M	*M
C+P	*M	*M
C+S	*M	*M
C+P+S	*M	*M

Table 29 C. cladosporioides: Germ tube length: Comparison between cultivars of antirrhinum for each location on the leaf (2day test)

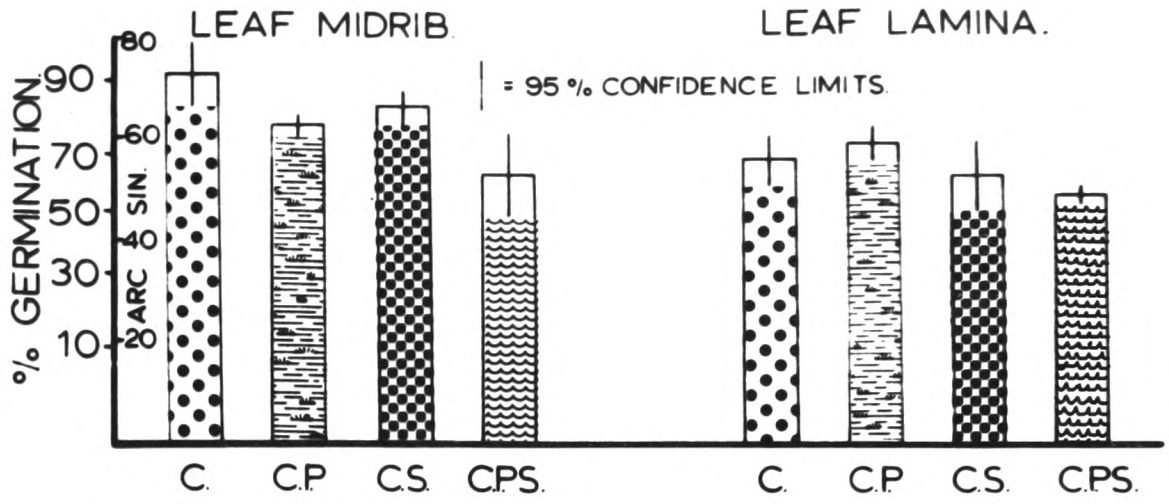
	Location on leaf	
Treatment	Midrib	Lamina
C	*F	*F
C+P	*F	-
C+S	*F	-
C+P+S	-	-

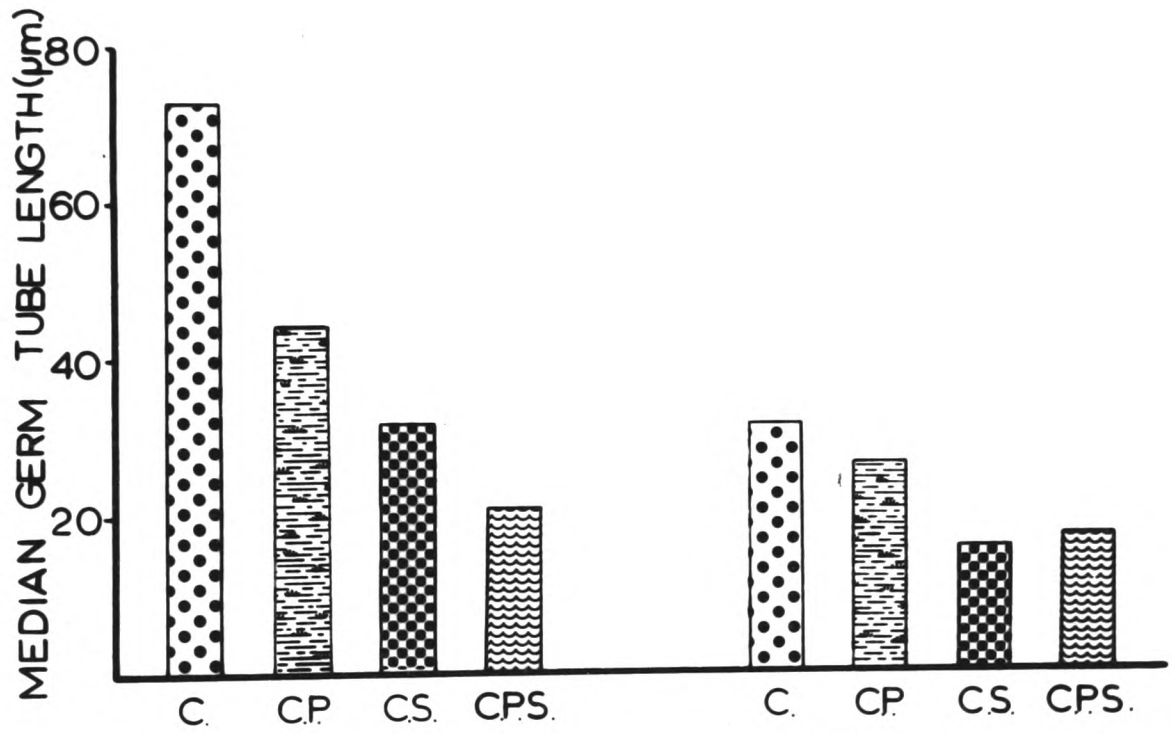
reduction in spore germination of C. cladosporioides was not significant on either area of the leaf.

The germ tube lengths of C. cladosporioides were reduced significantly ($p = .05$) by the presence of the other microbes on the leaf midrib. However, on the leaf lamina, a significant increase in germ tube length occurred in the presence of P. antirrhini alone, whereas there was a significant decrease in the other two cases. A comparison of each equivalent treatment on the leaf midrib with that on the leaf lamina (Table 28), showed that in all cases the median germ tube length on the leaf midrib was significantly ($p = .05$) larger than on the leaf lamina.

On leaves of A. Fi hybrid the percentage germination of spores (Fig. 41) was similar to that described above, except that here germination in the presence of P. antirrhini alone or with S. roseus on the leaf midrib was significantly ($p = .05$) lower than the control (C. cladosporioides alone). On the leaf lamina the germination in the presence of both other micro-organisms was significantly lower ($p = .05$) relative to the control. The spore germination of C. cladosporioides alone was significantly lower on the leaf midrib compared with the leaf lamina. (Fig. 42)

Germ tube length data show that, on the leaf midrib, all combinations of mixed cultures significantly ($p = .05$) decreased germ tube extension, but on the leaf lamina this reduction was only significant when S. roseus was present either alone or in combination with P. antirrhini. In all cases the equivalent treatments of micro-organisms resulted in significantly ($p = .05$) longer germ tube lengths on the leaf midrib as compared with the leaf lamina (Table 28).





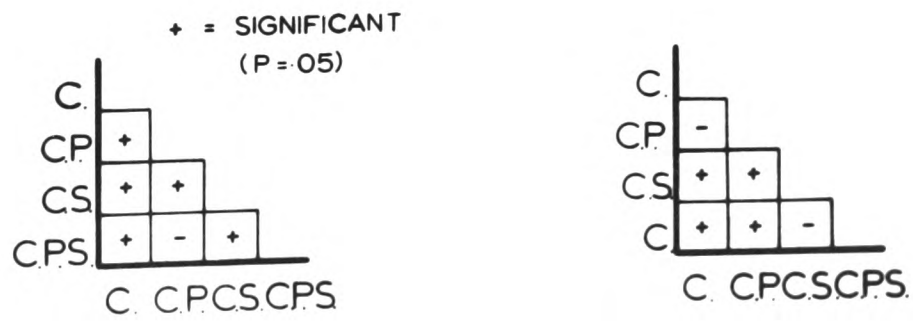


Fig. 42 C. cladosporioides: Interactions on detached leaves of A. Fi hybrid with single and mixed cultures of micro-organisms: Spore germination and germ tube growth (2 day test)

The comparison between antirrhinum cultivars of C. cladosporioides germ tube lengths for equivalent microbial treatments on the leaf lamina and midrib (Table 29) show that in all cases where a significant difference is recorded, leaves of A. Fi hybrid support the greater germ tube lengths, and that these differences occur in three out of four treatments on the leaf midrib but only one out of four treatments on the leaf lamina. In similar comparison for spore germination the observed differences were not significant in any case.

iii) S. roseus

On both cultivars of antirrhinum the presence of P. antirrhini led to a significantly ($p = .05$) higher volume of cells of S. roseus relative to the control (S. roseus alone), whereas the presence of C. cladosporioides led to a significantly lower volume of S. roseus cells (Fig. 43). The volume of cells produced when all three organisms were mixed is different on the two cultivars of antirrhinum. On A. Nanum a significantly higher volume of cells relative to the control was observed whereas the converse was observed on A. Fi hybrid.

A comparison of similar treatments of the two cultivars of leaf revealed that although the volume of S. roseus produced when incubated alone was significantly ($p = .05$) greater on A. Fi hybrid, the presence of C. cladosporioides alone or in combination with P. antirrhini gave significantly ($p = .05$) lower volumes compared to those on A. Nanum.

b) Six days incubation (18 hour 18°C days; 10°C nights)

i) P antirrhini

The mean values for percentage germination of uredospores varied markedly in each treatment (Fig. 44). On. A. Nanum leaves the only difference which was statistically significant ($p = .05$) was

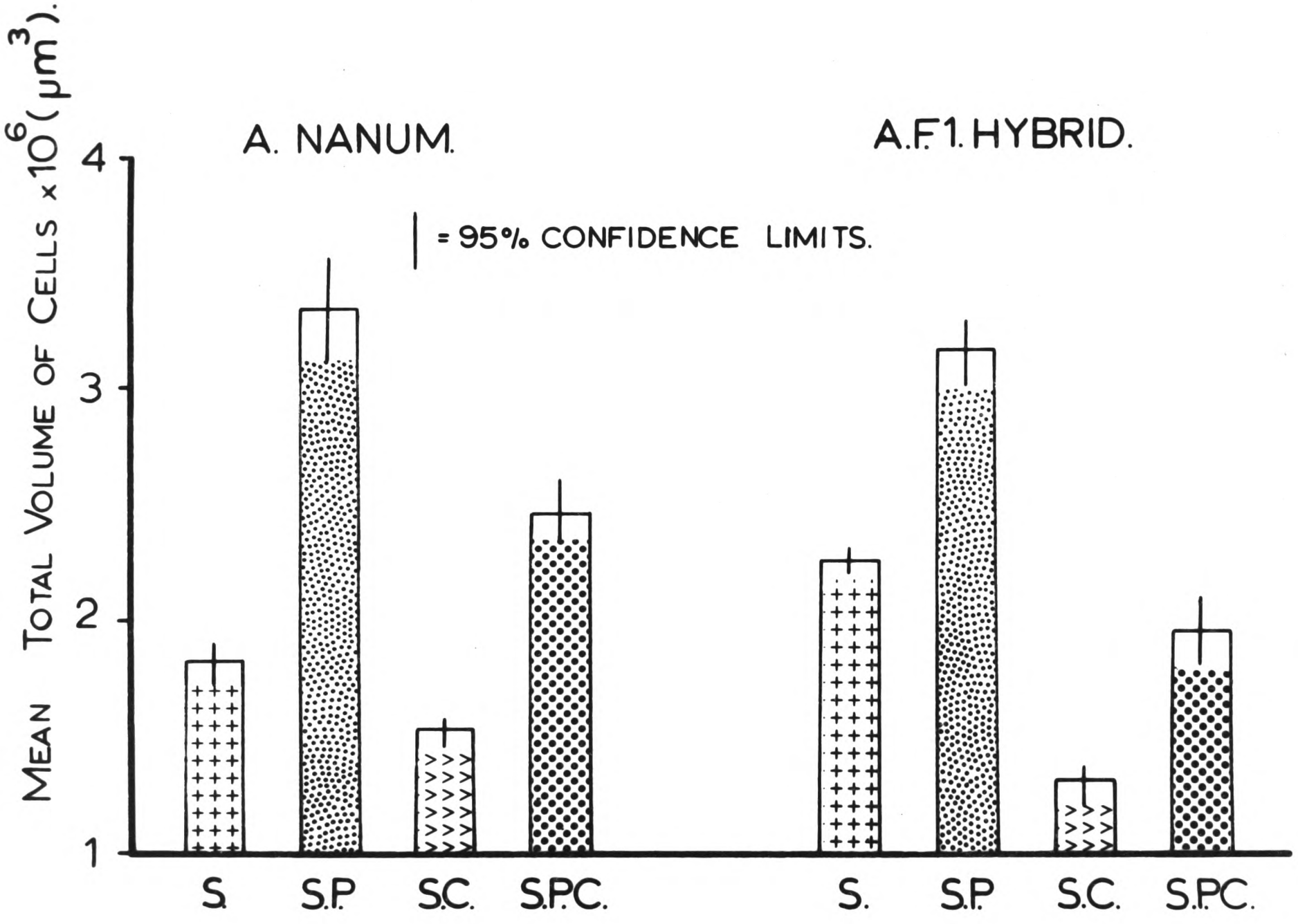


Fig. 43 S. roseus: Interactions on detached leaves with single and mixed cultures of micro-organisms: Mean total volume of cells (μm^3) per leaf (2 day test)

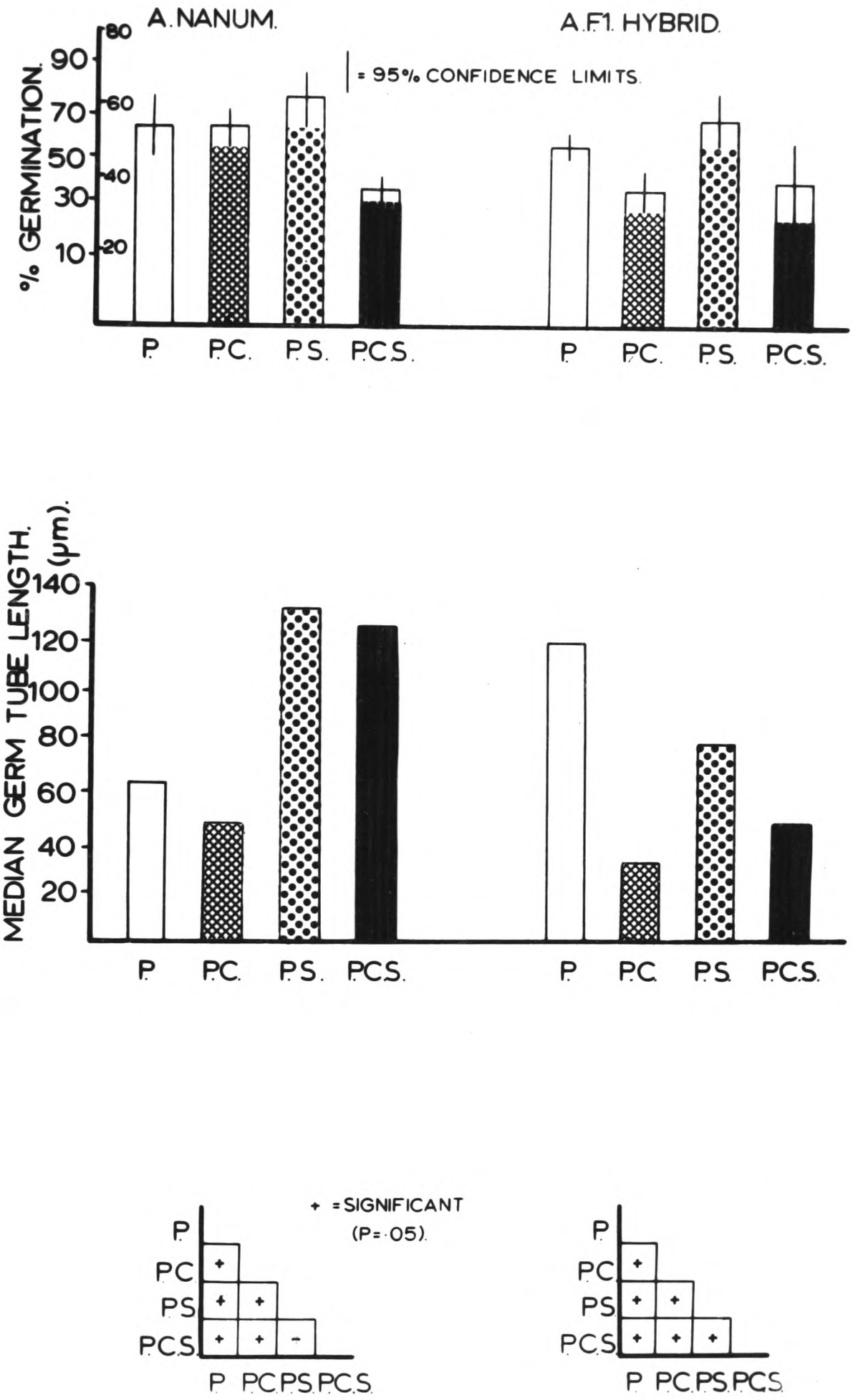


Fig. 44 P. antirrhini: Interactions on detached leaves with single and mixed cultures of saprophytic phylloplane micro-organisms: Spore germination and germ tube growth (6 day test)

the difference between the control (P. antirrhini alone) and the lower germination which occurred when all three organisms were incubated together. On leaves of A. Fi hybrid the germination of P. antirrhini was reduced significantly ($p = .05$) when it was incubated with C. cladosporioides alone. When comparison was made between the leaves of each cultivar of antirrhinum for equivalent treatments the only difference which was significant ($p = .05$) was the lower germination recorded in the presence of C. cladosporioides on A. Fi hybrid; 36.8% compared to 64.2% on A. Nanum.

The presence of C. cladosporioides led to a significant ($p = .05$) decrease in germ tube length of P. antirrhini on both antirrhinum cultivars. On A. Nanum the presence of S. roseus alone or in combination with C. cladosporioides led to a significantly ($p = .05$) greater median germ tube length, whereas on A. Fi hybrid leaves both of these combinations of micro-organisms and C. cladosporioides led to a reduction in the median germ tube length relative to the control values.

The comparison of equivalent treatments on the leaves of the two different cultivars of antirrhinum (Table 30) showed that all the differences between the leaf types were significant ($p = .05$). In the case of P. antirrhini alone the germ tube length was greater on A. Fi hybrid but that in all other cases the values of germ tube length were greater on A. Nanum.

On A. Nanum the presence of either or both of the saprophytes significantly reduced the incidence of leaf penetration by P. antirrhini and that these two saprophytes together reinforced one another to give a very low value of 1% penetration (Fig. 45). However, on A. Fi

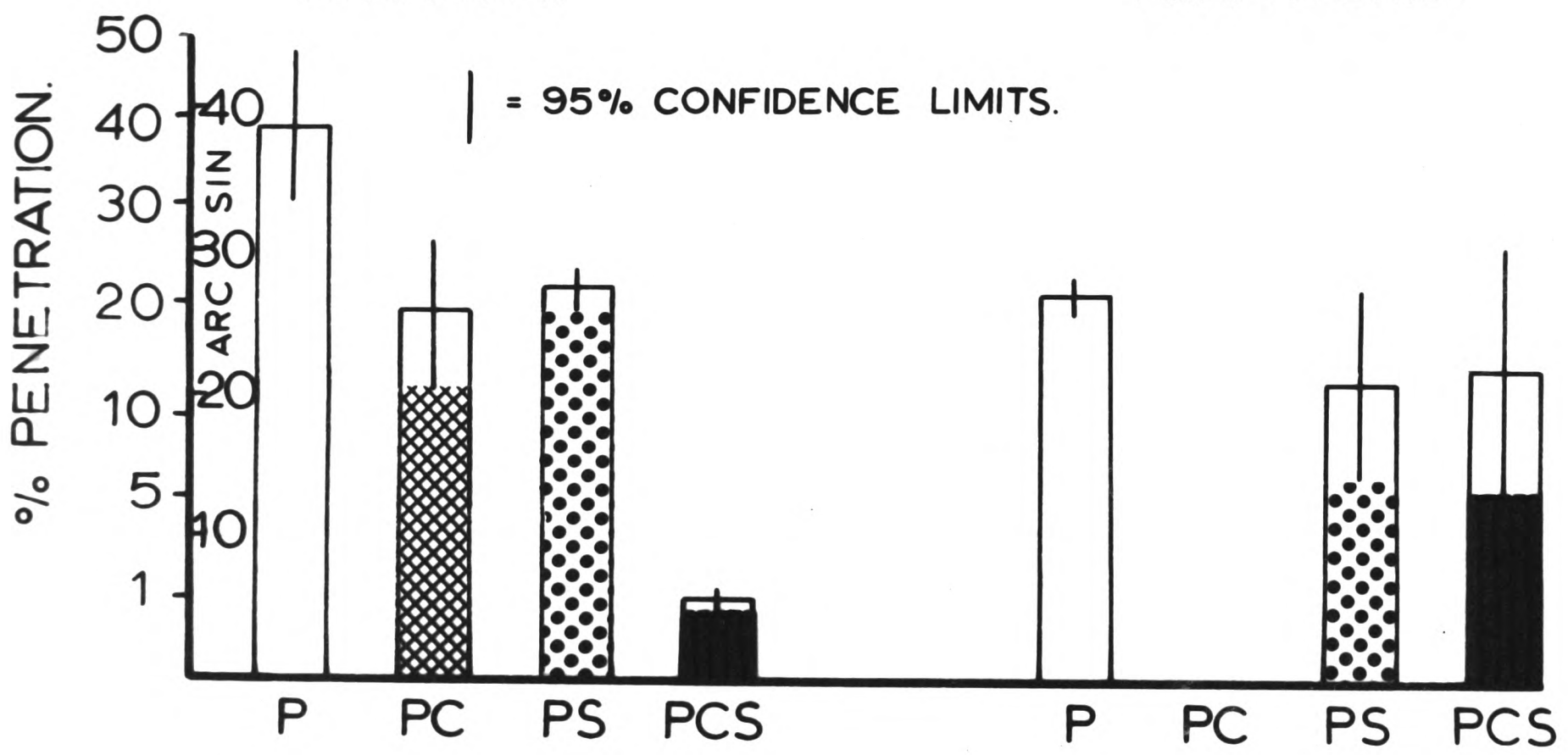


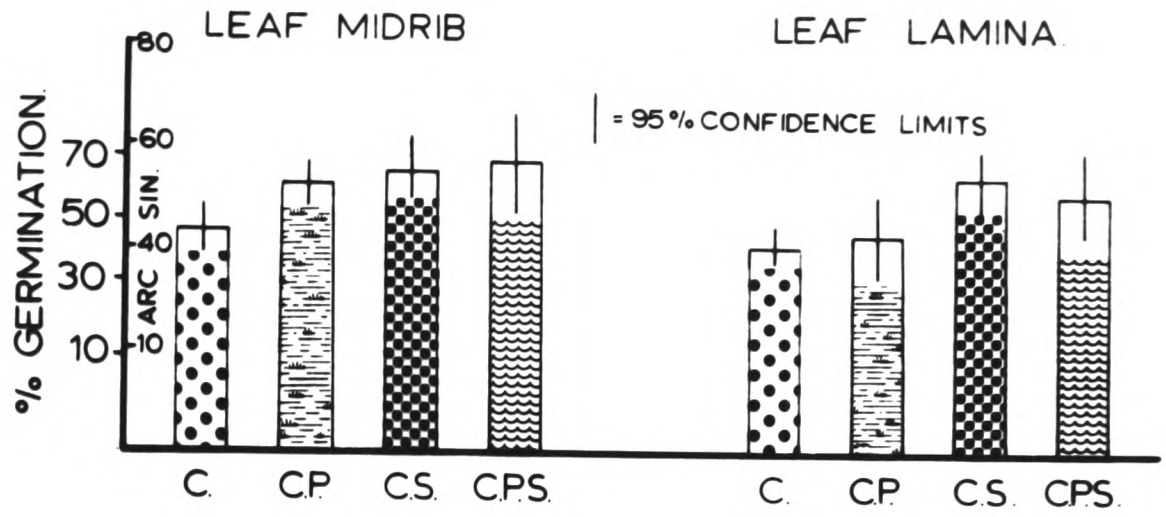
Fig. 45 P. antirrhini: Interactions on detached leaves with single and mixed cultures of saprophytic phylloplane micro-organisms: Leaf penetration (6 day test)

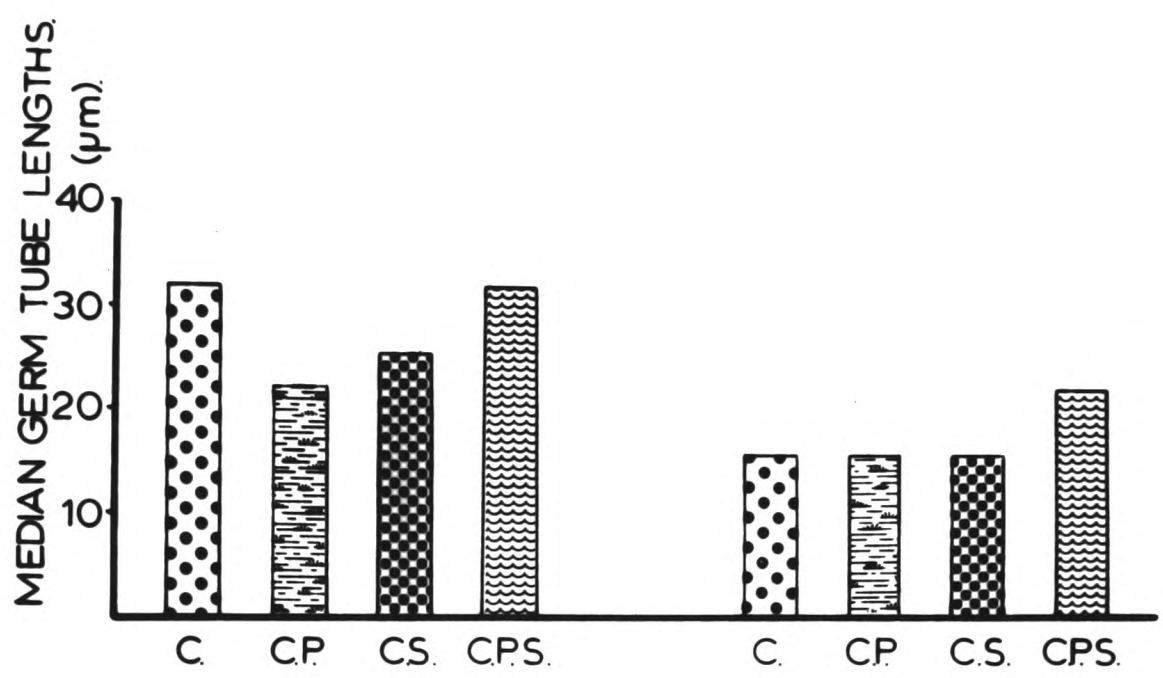
hybrid the presence of C. cladosporioides alone completely inhibited penetration of the leaves by P. antirrhini. Comparison between the cultivars of antirrhinum showed that the level of leaf penetration by P. antirrhini alone on A. Fi hybrid (18.1%) was significantly lower ($p = .05$) than the 39.1% observed on A. Nanum. The effects of C. cladosporioides on leaf penetration were significantly greater on A. Fi hybrid than on A. Nanum whereas the effects of S. roseus and C. cladosporioides together led to a significantly larger reduction in the penetration of leaves of A. Nanum compared with A. Fi hybrid.

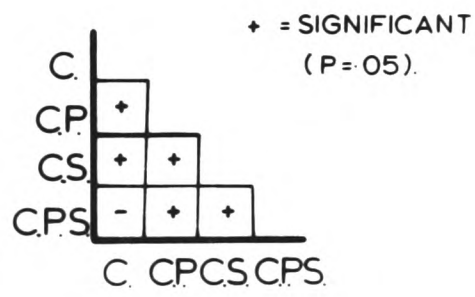
ii) C. cladosporioides

P. antirrhini or S. roseus alone significantly increased the spore germination of C. cladosporioides on the leaf midrib of A. Nanum whereas on the leaf lamina germination was significantly increased when S. roseus alone was incubated with C. cladosporioides (Fig. 46). In no case did equivalent combinations of organisms lead to significantly different spore germination in the different areas of the leaf.

The germ tube length data showed that on the leaf midrib the presence of P. antirrhini or S. roseus alone led to significantly ($p = .05$) shorter germ tubes compared with the C. cladosporioides control on the leaf midrib. However on the leaf lamina neither S. roseus nor P. antirrhini alone or in combination affected germ tube growth significantly ($p = .05$). Inspection of the matrices (Fig. 46) revealed that, in the presence of P. antirrhini, the germ tubes on the midrib were significantly shorter than those observed when C. cladosporioides was incubated with S. roseus alone. On the leaf lamina the median germ tube length of C. cladosporioides was significantly greater ($p = .05$) in the presence of the other two







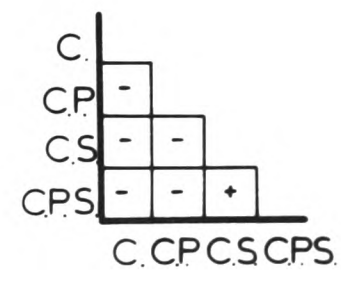


Fig. 46 C. cladosporioides: Interactions on detached leaves of A. Nanum with single and mixed cultures of micro-organisms: Spore germination and germ tube growth (6 day test)

Table 30 P. antirrhini: Germ tube lengths: Comparison between cultivars of antirrhini for each treatment (6 day test)

Treatment	P	P+C	P+S	P+S+C
Significance	*F	*N	*N	*N

Table 31 C. cladosporioides: Germ tube length: Comparison of samples from different locations (Midrib & Lamina) on leaves of both cultivars of antirrhinum (6 day test)

	Cultivar	
Treatment	Nanum	Fi hybrid
C	*M	*M
C+P	*M	*M
C+S	*M	*M
C+P+S	*M	*M

Table 32 C. cladosporioides: Germ tube length: Comparison of samples on different cultivars of antirrhinum for the same treatments on equivalent locations on the leaf

	Position on leaf	
Treatment	Midrib	Lamina
C	*F	*F
C+P	*F	*F
C+S	*F	*F
C+P+S	*F	*F

organisms together compared to the length attained in the presence of S. roseus alone.

Comparison of equivalent treatments on the leaf lamina and midrib (Table 31) showed that in all cases the observed median germ tube lengths on the midrib were significantly ($p = .05$) greater than on the leaf lamina.

The results of germination of C. cladosporioides spores on A. Fi hybrid (Fig. 47) showed that on the leaf midrib the presence of P. antirrhini or S. roseus alone gave a significantly higher ($p = .05$) germination than C. cladosporioides incubated in single culture. On the leaf lamina no significant differences in germination were observed between the different microbial treatments. Also the comparison of equivalent treatments on the two leaf areas showed that no significant differences occurred between the germination observed in these areas.

When C. cladosporioides was incubated with both the other organisms an increase in germ tube length occurred, relative to the control, on both the lamina and midrib (Fig. 47). Also on the leaf lamina, incubation with S. roseus or P. antirrhini alone led to a significantly ($P = .05$) shorter germ tube length compared with the control of C. cladosporioides alone.

Comparison of germ tube lengths resulting from equivalent treatments on equivalent locations on leaves of A. Nanum and A. Fi hybrid (Table 32) showed that in all cases the germ tube lengths were significantly ($p = .05$) larger on A. Fi hybrid.

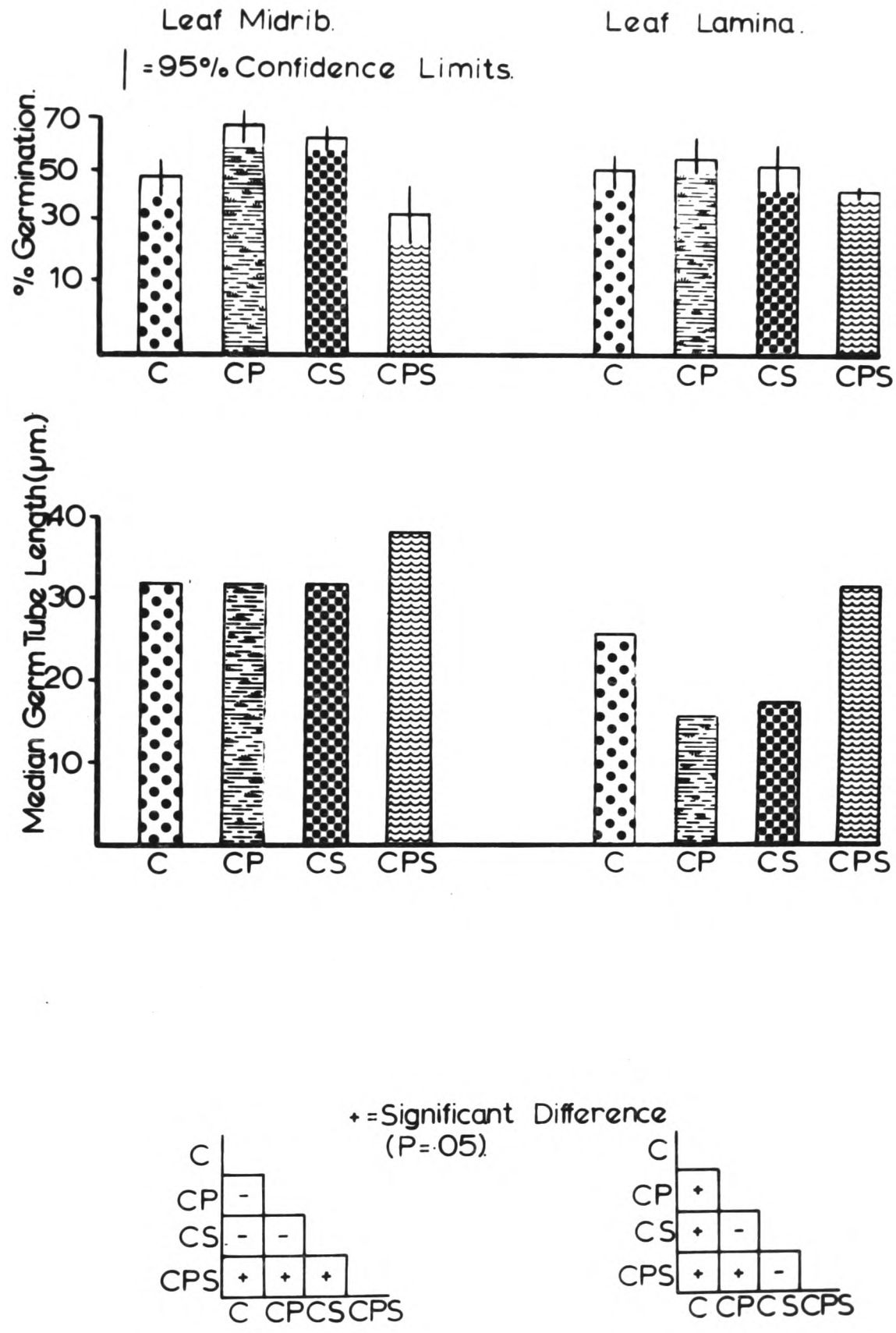


Fig. 47 C. cladosporioides: Interactions on detached leaves of A. Fi hybrid with single and mixed cultures of micro-organisms: Spore germination and germ tube growth (6 day test)

iii) S. roseus

Observations on the total volume of cells of S. roseus (Fig. 48) showed that a broadly similar pattern occurred for the different organism treatments on leaves of both cultivars of antirrhinum. When S. roseus was incubated with P. antirrhini on A. Nanum the total volume of cells of S. roseus was significantly ($p = .05$) higher than the control of S. roseus incubated alone. This was not true on A. Fi hybrid. However, the presence of C. cladosporioides led to significantly lower ($p = .05$) volumes of cells on both cultivars and values intermediate between these and the controls were obtained when all three organisms were incubated together. The volume of S. roseus cells in this mixture on A. Fi hybrid was significantly ($p = .05$) higher than the volume recorded for the same treatment of A. Nanum.

c) Comparison of two days and six days incubation

The comparison between the same mixtures of micro-organisms in the same positions on leaves of the same cultivar of antirrhinum subjected to the two different environmental regimes is given in the following sets of tables. These show if any observed difference in the parameter measured was statistically significant ($p = .05$), and, if so, then the appended figure 2 or 6 indicates the environment in which the measured parameter was greater.

i) P. antirrhini

Data for the germination of uredospores of P. antirrhini (Table 33) show that on A. Nanum the only significant difference between the treatments was in the sample in which all three organisms were incubated together. In this instance the two day sample gave a higher germination. On A. Fi hybrid the only significant difference ($p = .05$) was in the sample inoculated with

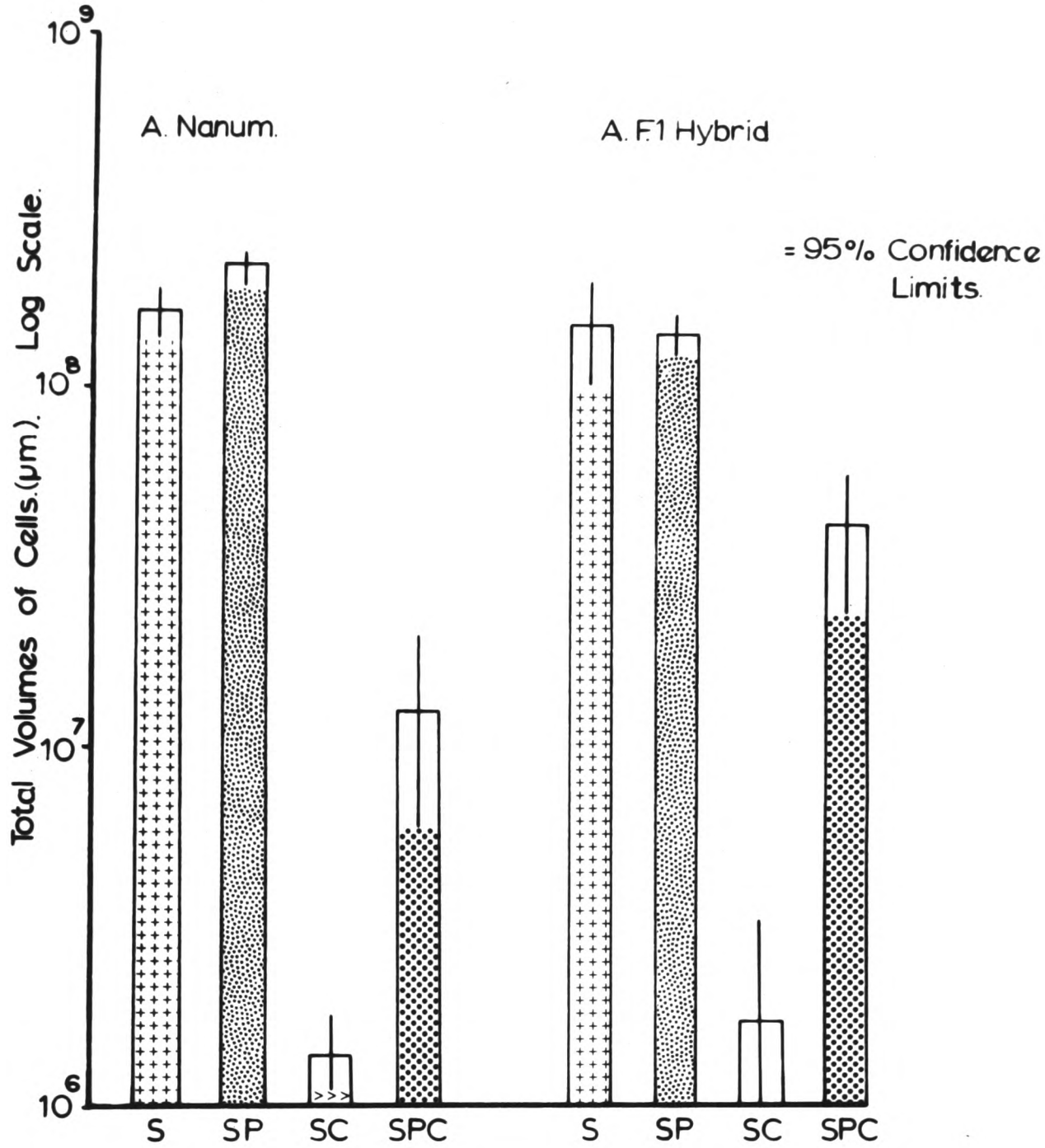


Fig. 48 S. roseus: Interactions on detached leaves with single and mixed cultures of micro-organisms: Mean total volume of cells per leaf (μm^3)

Table 33 P. antirrhini: Spore germination: Comparison of two and six day tests for equivalent treatments on the same cultivar of antirrhinum

Treatment	Cultivar	
	Nanum	Fi hybrid
P	-	*6
P+C	-	-
P+S	-	*2
P+C+S	*2	*2

Table 34 P. antirrhini: Germ tube length: Comparison of two and six day tests for equivalent treatments on the same cultivar of antirrhinum

Treatment	Cultivar	
	Nanum	Fi hybrid
P	*2	*6
P+C	*2	-
P+S	*2	*2
P+C+S	*2	*2

Table 35 P. antirrhini: Leaf penetration: Comparison of two and six day tests for equivalent treatments on the same cultivar of antirrhinum

Treatment	Cultivar	
	Nanum	Fi hybrid
P	-	*2
P+C	-	*2
P+S	-	*2
P+C+S	*2	-

C. cladosporioides alone, in which the two day sample had a higher percentage germination.

On A. Nanum the germ tube lengths of the two day test samples of P. antirrhini were significantly ($p = .05$) greater than the germ tube lengths of the six day test samples for all mixtures of organisms (Table 34). On A. Fi hybrid the incubation of P. antirrhini alone resulted in significantly ($p = .05$) longer germ tubes in the six day sample, but when the rust was incubated with S. roseus alone or together with C. cladosporioides, the germ tube lengths from the two day test were significantly greater.

On the leaves of A. Nanum the only significant difference between the two environmental regimes in terms of penetration of the leaf by P. antirrhini was observed when all three organisms were incubated together, when the two day test gave a higher percentage penetration (Table 35). However, on leaves of A. Fi hybrid the picture was different in that penetration was significantly higher in the two day test in all cases, except when all three organisms were incubated together, when no significant difference was observed between the tests in different environments.

ii) C. cladosporioides

Germination of C. cladosporioides spores on either location on A. Nanum leaves was significantly greater in the two day test when this fungus was incubated alone (Table 36). However, on A. Fi hybrid leaves, spore germination was significantly greater in the two day test for all treatments on the leaf midrib and when C. cladosporioides was incubated either alone, or with P. antirrhini, or with S. roseus and P. antirrhini together on the leaf midrib (Table 37).

Table 36 C. cladosporioides: Spore germination: Comparison of two and six day tests for equivalent treatments on the leaves of A. Nanum

Treatment	Location on leaf: Midrib	Lamina
C	*2	*2
C+P	-	-
C+S	-	-
C+P+S	-	-

Table 37 C. cladosporioides: Spore germination: Comparison of two and six day tests for equivalent treatments on the leaves of A. Fi hybrid

Treatment	Location on leaf: Midrib	Lamina
C	*2	*2
C+P	*2	*2
C+S	*2	-
C+P+S	*2	*2

Table 38 C. cladosporioides: Germ tube length: Comparison of two and six day tests for equivalent treatments on the leaves of A. Nanum

Treatment	Location on leaf: Midrib	Lamina
C	-	-
C+P	*2	*2
C+S	-	-
C+P+S	*6	*6

Table 39 C. cladosporioides: Germ tube length: Comparison of two and six day tests for equivalent treatments on the leaves of A. Fi hybrid

Treatment	Location on leaf	
	Midrib	Lamina
C	-	*2
C+P	*2	*2
C+S	-	-
C+P+S	*6	*6

Table 40 S. roseus: Total volume of cells per leaf: Comparison of two and six day tests for equivalent treatments on each cultivar of antirrhinum

Treatment	Cultivar	
	Nanum	Ki hybrid
S	*6	*6
S+P	*6	*6
S+C	-	-
S+P+C	*6	*6

C. cladosporioides germ tube lengths on both locations on A. Nanum leaves were significantly larger in the two day test when C. cladosporioides and P. antirrhini were incubated together and in the six day test when all three microbes were present (Table 38). Similar results were obtained on A. Fi hybrid leaves and moreover C. cladosporioides germ tube lengths were significantly greater in the two day test when this fungus was incubated alone on the leaf lamina (Table 39).

iii) S. roseus

The pattern of significant differences between the two and six day tests in terms of total volume of cells of S. roseus (Table 40) were the same for both cultivars of antirrhinum, namely, that when S. roseus was incubated alone, with P. antirrhini and with P. antirrhini plus S. roseus, the volume of cells observed in the six day test was significantly greater than that observed in the two day test.

DISCUSSION

The differential effects of antirrhinum leaf leachates on the pathogenic and saprophytic micro-organisms used in the *in vitro* tests were most marked. The pronounced inhibition of uredospore germination and germ tube growth of *P. antirrhini*, produced by the leachates from A. fi hybrid, was contrasted by the general stimulation of growth of both saprophytic organisms. The ability of host leaf exudates to inhibit the spore germination of pathogenic fungi has been observed by several workers (Hafiz, 1952; Topps & Wain, 1957; van Velson, 1957; Kono Akitsma, 1960; Singh, 1965; Sharma & Sinha, 1971). The degree of susceptibility of the host plant has been shown to affect this inhibition and the greatest inhibition has been observed in washings from younger leaves (Sharma & Sinha, 1971). While the results presented here support the findings that the inhibition of the pathogen is affected by differences in varietal susceptibility, no differences were observed between the leachates from young and old leaves. However, the stimulation of germ tube growth in *C. cladosporioides* was affected by differences in leaf age as well as plant variety. The variation in stimulation would seem to bear a marked correlation to the quantities of carbohydrates present in the leachates. The highest carbohydrate levels (p. 107) and the greatest germ tube extension occur in leachates from the older leaves of A. Nanum (Table 23). Otherwise the stimulation of saprophytes seems to take the form of an increase in the initial rate of spore germination in *C. cladosporioides* within the first day, and an increase in the total volume of cells of *S. roseus* over a two day period. Similar effects

had been observed previously when these organisms had been incubated in solutions containing sugars (p.72,74).

Unfortunately the results of the colonisation of attached leaves on whole plants gave very variable results, even under the conditions maintained in controlled environment rooms. However, other workers have also observed great variability in the increase of bacterial populations from known inocula on whole plants in controlled environment conditions (Leben et al., 1968). Similar variable results were obtained in experiments using S. roseus. In many cases the yeast cells failed to maintain themselves on sterile larch seedlings in closed flasks. This was ascribed to the lack of moisture at the surface of the plants (McBride, 1970). However, in the present study the use of detached leaves seems to have reduced the variability between replicates sufficiently to allow some patterns of leaf colonisation to be discerned.

Experiments on S. roseus show that not only was there an overall increase in the total volume of cells on leaves of all ages of both cultivars of antirrhinum, but also that this increase was only of the order of x 30 on younger leaves as opposed to x 170 to x 240 on older leaves. The difference between younger and older leaves reached significance from four days after inoculation on A. fi hybrid and seven days after inoculation on the leaves of A. nanum. This large difference is in agreement with field studies, using the sporefall technique, on the colonisation of wheat leaves by S. roseus in which few yeast cells were isolated until the leaf had lived for over 50% of its expected life. Both the humidity of the surrounding atmosphere and leaf age were found to affect the colonisation of

leaves by this yeast (Last, 1955a). In the experiments described here the sporefall technique only yielded positive results on and after the fourteen day sample. This might suggest that some period of establishment is necessary before ballistospores could be produced by S. roseus. In field grown antirrhinums, the sudden increase in numbers of S. roseus isolated by the sporefall method lagged up to fourteen days behind the marked increase in numbers observed by a leaf washing technique, which would appear to agree with the observations on detached leaves. The more or less immediate production of secondary spores by spores of S. roseus has been observed in vitro (Buller, 1933), but it might well be that normal yeast cells exhibit some form of time lag before producing ballistospores. Such a time lag might possibly allow time for colony formation as numerous groups of yeast cells were seen in the depressions over the anticlinal cell walls of the leaf epidermis from the fourteen day sample. Similar patterns in the distribution of yeast cells have been observed on the leaves of other plants (Last, 1955a).

On all antirrhinum leaves the presence of C. cladosporioides exerted a marked effect on the increase of S. roseus. On older leaves the volume of cells was significantly lower in the sample on day one and subsequently. However, on the younger leaves the pattern is not the same, in that on young A. Nanum leaves the difference in volume of cells of S. roseus was significantly less after seven days, but no such consistent pattern was observed in the results on the younger leaves of A. Fi hybrid. The antagonism between S. roseus and C. cladosporioides has also been observed in plate cultures (Last, 1955a). This antagonism will be discussed in more detail

later.

The study of the colonisation of leaves by C. cladosporioides was somewhat different in that it made use of direct microscopic observations. Some initial experiments, not described here, gave rise to an idea that the veinal regions of the leaf, especially the midrib, stimulated the development of C. cladosporioides to a greater extent than the leaf lamina. The veinal distribution of Cladosporium spp. and other fungi has been observed on sycamore leaves (Pugh & Buckley, 1971a). In these experiments separate estimates on spore germination and germ tube length were made for leaf midrib and lamina. Examination of the figures for germination shows that, although in samples from older leaves the mean germination on the leaf midrib was higher than on the leaf lamina for the first four days of incubation, only in the case of older leaves of A. Nanum on the first day sample was the difference in means significant ($p = .05$). It was possible that the overall difference in germination recorded on individual leaves was sufficiently great to yield overall mean values with confidence limits sufficiently wide to mask any effects of leaf position which might be apparent on an individual leaf. A reappraisal of the raw data on spore germination for the three replicate experiments for each leaf type showed that on the older leaves during the first four days all replicates (15/15) gave higher germination on the leaf midrib compared to the lamina at each sampling. On younger leaves between 5/15 and 8/15 leaves showed this effect. If there was no difference in spore germination on the different areas of the leaf one would expect that each area should support a higher germination for about 50% of the samples taken. The use of the 'sign' test (Campbell, 1967) shows that for the older

leaves spore germination was significantly ($p = .01$) greater than one would expect by chance alone and that different areas of the leaf affect germination in different ways. This enhancement, on the leaf midrib, was observed both when C. cladosporioides was incubated alone and when S. roseus was present as well. Comparison of mean germ tube lengths on individual leaves showed that, for samples after one or two days incubation, germ tube growth was enhanced on the midrib area of older leaves of both cultivars of antirrhinum. Later confirmation of this difference was obtained in experiments on the interaction of micro-organisms on older (LPI 1.5) leaves. Germ tube length data was analysed statistically and in both two day (Fig. 28) and six day tests (Fig. 31), the germ tube lengths of C. cladosporioides on the leaf midrib were significantly greater ($p = .05$) than those growing on the leaf lamina of both antirrhinum cultivars. Thus, it was reasonable to suggest that spore germination and germ tube growth were enhanced on the leaf midrib, relative to the leaf lamina, especially during the first few days of incubation. Although this effect may be observed on individual leaves, there was sufficient variability in the development and growth of C. cladosporioides to mask this effect in the cumulative data from the leaf colonisation experiments.

Several reasons for the veinal distribution of fungi on field plants have been suggested (Pugh & Buckley, 1971a), but of these the factor which might have accounted for the major part of the effect in this case was the presence of more exudates over the veins (Pesante, 1963) which might have been due to the activity of vein extension cells in bringing nutrients to the surface along the veins (Wylie, 1943). As mentioned above, the presence of nutrients stimulated

both the rate of spore germination and germ tube growth of C. cladosporioides. The lack of an observed 'midrib effect' on young leaves (LPI 0.5) was consistent with the morphology of these leaves in which the majority of the vascular network of the leaf was present. Subsequent leaf growth was mainly due to cell expansion (CH 2). Thus in younger leaves no part of the leaf lamina would be sufficiently distant from veinal material to allow any differential nutrient leaching to occur especially at the lower level of leaching observed in younger leaves (p.107).

In samples of leaves of all ages a high initial rate of germination was observed. A similar high initial germination rate was observed for C. herbarum on barley leaves (Skidmore & Dickinson, 1973). This rapid germination rate might suggest that in cases where large numbers of non germinated spores have been observed on field plants during late spring and early summer (Dickinson, 1965; McBride, 1970; Pugh & Buckley, 1971a; Bainbridge & Dickinson, 1972), the environmental conditions, especially relative humidity, were limiting. However it would be unrealistic to assume that conditions in late summer would have been consistently wetter in all the sampling sites used by those workers in different years. As the leaf ages it becomes more susceptible to leaching (Cholodny, 1932) and the nutrient content of exudates may increase as has been observed in larch (McBride, 1970) and in antirrhinum (p.107). As the rate of germination of C. cladosporioides has been shown to increase in the presence of nutrients (p. 72), it may be that the spores are able to germinate more readily in field plants when high humidity conditions necessary for germination (p. 68) (Stott, 1971) occur for short periods. Some recent work has confirmed that such

bursts of activity in favourable climatic conditions are enhanced when the nutrient status of the leaf surface was increased (Diem, 1974).

After the initial period the patterns of germination were different for the two ages of leaves studied. On younger leaves the percentage germination remained more or less constant throughout the period of the experiments. However, on the older leaves germination increased to a peak value at four or five days, depending on cultivar and location on leaf, and then declined. Sporulation was observed four or five days after inoculation, and this production of secondary spores might explain the observed decline in overall germination. The sporulation of Cladosporium spp. on green leaves is not peculiar to these experiments, as it has been observed after only 48 hours incubation at 15-20°C on barley leaves in high humidity conditions (Skidmore, 1974; Diem, 1974). Sporulation has also been observed on green leaves of halimione (Dickinson, 1965), larch (McBride, 1970) and sycamore (Pugh & Buckley, 1971a) in the field.

The growth patterns of C. cladosporioides germ tubes on detached leaves were initially similar to those observed for spore germination in that after an initial rapid rate of growth during the first 24 hours little increase in growth was observed during the next six days. After that time the germ tube length did show a continuing increase in length until the end of the experiment. This increase was of about x 2 on younger leaves of both cultivars, x 6 on older leaves of A. Fi hybrid and x 7.5 on older leaves of A. Nanum. The difference in these increases may reflect the differences in the nutritional status of leaves of different cultivars as a similar pattern of increase in germ tube length was observed in the two day period of the in vitro experiments on the effects of leaf leachates on C. cladosporioides

(Table 23). Also, these differences correlate well with the difference of total carbohydrates found in the leachates from young and old leaves of the different cultivars of antirrhinum (Table 17). However, the lack of germ tube growth correlated to leaf age has been observed in the restriction of mycelial development on young leaves (Dickinson, 1967), in which the production of antifungal substances by the leaves may be implicated. The ability to form phytoalexins has been shown to decrease with age, especially with the onset of senescence (Bailey, 1969). However, the rooted detached leaves used in these experiments remained green throughout the period of the experiment and no visible signs of senescence were observed.

On older leaves the effect of S. roseus on the germination of C. cladosporioides was a general reduction in germination during the first few days of incubation. The reduction was significant ($p = .05$) only on the midrib of A. Fi hybrid. However the patterns of germination were consistent for both locations on the leaf of both antirrhinum cultivars. The peak germination was not attained until two days later than in cultures of C. cladosporioides alone. After this time the percentage germination in the mixed culture was consistently higher than that for C. cladosporioides alone. One possible explanation for this could be that the numbers of secondary spores produced might be lower, although the overall germination would appear to be higher. Quantitative estimates of the numbers of sporulating conidia were not made as the fragile nature of the spore chains meant that these tended to break during the microscopic preparation. Nevertheless, marked clumps of small spores were seen around the ends of structures which could easily have been conidio-conidiophores and more complete sporulating conidia were observed.

On the younger leaves the mean percentage germination in the mixed cultures was consistently lower than that in the cultures of C. cladosporioides alone. Although the confidence limits around the means would indicate that the difference was not significant on a quantitative basis, some qualitative effect might be shown. If there was no difference in germination between these treatments, then one would expect that at least some of the values of the means for the mixed cultures would be higher than those for C. cladosporioides alone. In these experiments all eight means for the mixed cultures are lower on both locations on both cultivars of leaves and application of the 'sign' test (Campbell, 1967) would give a 95% probability of being correct in the rejection of the null hypothesis. A similar argument might be applied to the germ tube length data obtained on the older leaves of both varieties of antirrhinum in which the mean germ tube length for the mixed inoculum was consistently lower than for C. cladosporioides alone. No consistent effect was observed on younger leaves.

The reduction of germination and germ tube length of C. herbarum by S. roseus has been observed in vitro (McBride, 1970). In the in vitro experiments in this study (Fig. 36) S. roseus reduced germ tube growth significantly ($p = .05$). It has been demonstrated that S. roseus produces antifungal substances (Yamasaki et al., 1951) and so the observed inhibition of germination and germ tube growth may have been the result of chemical antagonism. However, it is also possible that inhibition arose from the competition of the yeast and filamentous fungus for nutrients. Also, many fungal spores have been shown to lose nutrients very rapidly by leaching (Ko & Lockwood, 1967), which may have occurred

both *in vitro* and on the leaf surface.

The antagonism between *S. roseus* and *C. cladosporioides* was not one sided in that *S. roseus* was inhibited by the other fungus. The rapid overgrowth of *S. roseus* by *Cladosporium* spp. in plate culture has been observed (Last, 1955a). Also *Cladosporium* spp. have been observed to inhibit fungi on lettuce (Newhook, 1951b), strawberries (Bhatt & Vaughan, 1962), barley (Diem, 1969a), beans (van den Heuval, 1970) sugarbeet (Warren, 1972b) and several other plants. On barley this antagonism has been shown to be due to nutrient competition (Diem, 1969b). It may well be that nutrient competition occurs on the leaf surface of antirrhinums although, under low nutrient conditions *in vitro*, *S. roseus* shows no signs of inhibition (Fig. 38) which could be due to the leaching of nutrients from fungal spores under those conditions. Another possibility is the production of antifungal substances by *C. cladosporioides*. Such substances have been implicated in the control of *B. cinerea* by *Cladosporium* sp. (Bhatt & Vaughan, 1963). However unless a nutrient is required for production of such substances, it is odd that in *in vitro* experiments no antagonistic effects of *C. cladosporioides* on *S. roseus* were observed. An alternative hypothesis is, that since *Cladosporium* sp. have been shown to produce substances affecting the plant (Valadon & Lodge, 1970), it may be that some substances produced could trigger phytoalexin production (Bailey, 1971). However, while this might explain why *S. roseus* was inhibited on leaves in the presence of *C. cladosporioides*, it would not explain why the latter fungus was inhibited on the leaf by *S. roseus*, as it would be reasonable to expect any such phytoalexin to operate against *C. cladosporioides* when incubated alone on the leaf surface.

The interactions between P. antirrhini, C. cladosporioides and S. roseus present a somewhat complex picture in whibh it was not easy to discern consistent patterns of antagonism or synergism. When optimum conditions for the germination of P. antirrhini as predicted by in vitro spore germination tests, were used to incubate cultures on detached leaves, the effect on the rust fungus was similar to that observed in the in vitro tests on spore germination in leaf leachates. Spore germination, germ tube growth and leaf penetration were significantly lower on A. Fi hybrid although spore germination on this cultivar was higher than predicted by the in vitro experiments, as was the germ tube growth on A. Nanum.

However, the effects of the saprophytic organisms on the pathogen were not the same as those predicted by the in vitro tests. These tests indicated that S. roseus was antagonistic and C. cladosporioides was synergistic towards the rust, whereas in the two day test on A. Fi hybrid leaves rust germ tube growth and leaf penetration were stimulated in the presence of S. roseus (Fig. 40), on A. Nanum rust germ tube growth was significantly reduced in the presence of C. cladosporioides. The six day test complicates the issue still further, for although the presence of C. cladosporioides reduced germ tube growth on both cultivars of antirrhinum and germination on A. Fi hybrid, germ tube growth was enhanced on A. Nanum and reduced on A. Fi hybrid in the presence of S. roseus. Leaf penetration was significantly reduced on both antirrhinums by C. cladosporioides. Although a reduction in leaf penetration was observed in the presence of S. roseus only on A. Fi hybrid leaves was this significant.

Differences in the interactions between micro-organisms *in vivo* and *in vitro* have been observed in the inhibition of *Alternaria zinniae* by *Aureobasidium pullulans* in which no inhibition was observed *in vitro* but a definite inhibition occurred *in vivo* (van den Heuvel, 1969). Similar differences between *in vitro* and *in vivo* tests were observed in the interaction of *Candida* sp. with *Cochliobolus miyabeanus* on rice leaves (Akai & Kuramota, 1968). Bailey (1971) suggested that these workers results indicated the formation of inhibitory substances by the plant in response to the antagonistic organism. Such a hypothesis might be relevant in this case as, although *C. cladosporioides* was not shown to be antagonistic *in vitro*, this micro-organism did show antagonism towards *P. antirrhini* on the leaf. This was not very obvious in the results of the two day test in which conditions were optimal for the germination of the rust. In this case there was some evidence of enhancement of germination on leaves of A. Fi hybrid. Nevertheless, in the six day test *C. cladosporioides* did show antagonism towards rust spore germination on A. Fi hybrid and towards germ tube growth and leaf penetration in both cultivars. These results might indicate either a delay in host response or the inability of any antifungal compounds formed to inhibit rust spore development when conditions were optimal for this organism. These ideas would seem unlikely in that phytoalexin production is thought to be fairly rapid (Cruickshank & Perrin, 1961) although the level of phytoalexin might not have built up to a sufficiently high level in two days. However, phytoalexins have been shown to be far more active against non pathogens than pathogens (Cruickshank, 1962) and so if *C. cladosporioides* triggered the phytoalexin it should not grow well on the leaf surface when incubated alone. However, germination, germ tube growth and

sporulation have all been observed on leaves of this physiologic age (LPI 1.5) (Figs. 33, 35), and so it would seem unlikely that the antagonism of P. antirrhini by C. cladosporioides on the leaf surface was solely due to phytoalexin formation. Also, P. antirrhini is antagonistic to C. cladosporioides both in vivo and in vitro, indicating that this reaction was unaffected by the leaf.

The antagonism of D. sorokiniana by Cladosporium sp. has been shown to be due to competition for nutrients (Diem, 1969b). It is difficult to see how such an explanation could fit the antagonism between C. cladosporioides and P. antirrhini in this study as neither organism has a requirement for nutrient for germination, nor does the addition of nutrient to uredospores of P. antirrhini affect germ tube growth. Also uredospores have been shown to use endogenous nutrient sources during germination (Shu et al., 1956). Thus, one would have to propose that C. cladosporioides germinated sufficiently quickly and close enough to the rust spores to leach out sufficient nutrient to reduce germ tube growth. This might explain the consistent antagonistic effects observed in the six day test. If this was solely the case, then it would be probable that some form of nutrient competition would occur between S. roseus and P. antirrhini, but this was not observed as both germ tube growth and leaf penetration were enhanced on A. Fi hybrid in the two day test. The effects of S. roseus on P. antirrhini did not give a clear pattern in the six day test, as on A. Nanum germ tube growth was stimulated and leaf penetration reduced, whereas on A. Fi hybrid, although germ tube growth was significantly ($p = .05$) reduced, the observed reduction of leaf penetration was not significant.

S. roseus was affected by the presence of the rust. A significant increase in the total volume of cells was observed in the two day test on both varieties of antirrhinum, and also in the six day test on A. Nanum leaves. An increase in the volume of cells was also observed in the in vitro tests when P. antirrhini was present. Similar increases in the numbers of S. roseus have been observed in later stages of rust infection on mint (Last & Deighton, 1965) and antirrhinum (Last, 1970). Also increases in the numbers of S. roseus were observed shortly after antirrhinum plants had been inoculated with uredospores of P. antirrhini in the field (Fig. 5).

The antagonism between S. roseus and C. cladosporioides seen in the colonisation experiments was observed in these experiments. When all three organisms tested were incubated together, then the germ tubes of P. antirrhini were of a length intermediate to the lengths observed when the rust was incubated with each saprophyte separately. This antagonism between the saprophytes reduced their individual effect on P. antirrhini. A similar comparison for germination or leaf penetration did not yield such consistent results.

While it has been observed that the materials leached from antirrhinum leaves, especially A. Fi hybrid, inhibited the growth of P. antirrhini, the two saprophytic micro-organisms were stimulated by these leachates. The saprophytic organisms were able to colonise green leaves and showed mutual antagonism when in mixed culture on leaves. The interaction between S. roseus and C. cladosporioides in vitro was similar to that observed on leaves. However, interactions between these organisms and P. antirrhini in vitro were almost the converse of those observed on the leaf surface, in which

C. cladosporioides was antagonistic, while the presence of S. roseus appeared to enhance the growth of the rust in some cases. Thus it would appear that the interactions between saprophytes and pathogens on the leaf surface are complex and it is necessary to develop techniques to enable further detailed studies to be done to elucidate the precise mechanisms underlying these interactions.

PART II

PHYLLOPLANE MICROFLORAS OF OTHER SPECIES

PART II

PHYLLOPLANE MICROFLORAS OF OTHER SPECIES

INTRODUCTION

Bier (1965) and McBride (1969, 1970) both demonstrated that interactions did occur between saprophytic micro-organisms isolated from aerial surfaces of tree species and pathogenic micro-organisms which could infect these plant surfaces. Moreover, they showed that some of these saprophytes could exert some measure of control against the pathogens. As relatively little work has been done on the phylloplanes of tree species, in particular into their interactions with pathogens it seemed appropriate to pursue this field of research.

Two tree species were found, within a reasonable distance of Edinburgh, to be infected by specific leaf pathogens. These were *Picea abies* Karsten infected by *Chrysomyxa abietis* Unger (Plate 18) and *Acer pseudoplatanus* L. infected by *Rhytisma acerinum* (Pers) Fr. (Plate 19). An examination of the literature revealed that there were no reports on the phylloplane microflora of Norway spruce but there was a detailed study of filamentous fungi and ballistospore forming yeasts found on the phylloplane of sycamore (Pugh & Buckley, 1971a). Most reports on either of the pathogens are concerned with epidemiology, cytology and life history descriptions although in the case of *R. acerinum* some observations have been done on the infection of various *Acer* spp. (Massee, 1901). The work described here was done mainly in 1971.

It was intended to observe any interactions which might occur between saprophytic phylloplane micro-organisms of these tree species

Plate 18. Chrysomyxa abietis on needles of Norway spruce (Picea abies): Castle O'er Forest, Dumfriesshire, May 1971.

Plate 19. Rhytisma acerinum on leaves of sycamore (Acer pseudoplatanus): Newbattle Abbey, Midlothian, September 1971.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and the relevant specific obligate leaf pathogen. Some of the saprophytes isolated, together with the appropriate leaf pathogen would be used as components in a model system to observe in detail the interactions between saprophyte, pathogen and host plant. Neither of the host pathogen combinations proved to be amenable to study under controlled conditions and so the work described here concerns the isolation and enumeration of saprophytic micro-organisms from the phylloplane of both tree species. Special attention was paid to any changes which might occur in the saprophytic phylloplane population as a result of infection of the leaves by a pathogen. Also experiments on the germination of spores of the pathogenic fungi and infection trials are described.

REVIEW OF LITERATURE

(a) PHYLLOPLANE MICROFLORAS OF SOME TREE SPECIES

The study of saprophytic micro-organisms on leaves of trees has not received as much direct attention as have studies on other higher plants, but it is possible to amass a considerable body of information from work which may not have had the study of phylloplane micro-organisms as its major topic.

Chesters (1949) considered that the aerial parts of many plant shoot systems are 'worked over' by weak parasitic and saprophytic species, including a whole galaxy of fungi, so that the plant parts arrive at the soil surface very much depleted of their nutritional possibilities. However he considered that leaves of trees and shrubs were an exception and that these leaves reached the soil surface little changed by pre-attack by fungi, although in some cases

fungi were present. This hypothesis may have been taken up during the fifties and early sixties by some of the workers who were studying the breakdown of tree leaf litter by fungi. While they devoted little time to a detailed study of the microflora of living leaves, nevertheless, the majority of workers did carry out some examination of fungi on leaves prior to leaf fall. A list of tree species worked on by various authors, investigating litter decay, is given (Table 41).

Observations on the presence of certain fungi prior to leaf fall may pose difficulties in that many species were stated to be primary colonisers of leaves only by virtue of the fact that they were present on leaves before these reached the ground. Some of these fungi may have colonised already dead or senescent leaves rather than living leaves. Kendrick and Burges (1962) considered that A. pullulans and Fusicoccum bacillare Sacc. and Penz. inhabit the needles of Pinus sylvestris L. which were already senescent and fell to the ground during the autumn needle fall. Hayes (1965) stated that Aspergillus fumigatus Fres. Halplographium penicillioides Fautrey (Now Thysanophora penicillioides (Roum) Kendrick comb. nov.) and Penicillium frequentans Westling were present at leaf fall or arrived very shortly afterwards in Abies grandis Lind., Picea sitchensis (Bong) Carr, and P. sylvestris. Macauley and Thrower (1966) found Protestega eucalipti Cooke and Massee, Cherbarum, Readeriella mirabilis Syd., Alternaria tenuis, and Epicoccum spp. as primary colonisers on fallen leaves of Eucalyptus regnans F. Muell. Gremmen (1959) gave no indication whether the fungi which he isolated were from dead or living leaves. Thus there is some confusion as to whether these primary colonisers were present on

Table 41. References of tree species used in studies on leaf litter decomposition

Species	Reference
Abies grandis Lindley	Hayes, 1965
Amelanchier sp.	Smit & Wieringa, 1953
Betula pubescens Ehrh.	Hering, 1965
Corylus avellana L.	Hering, 1965
Eucalyptus regnans F. Muell.	Macauley & Thrower, 1966
Fagus sylvatica L.	Smit & Wieringa, 1953
Fraxinus sp.	Hering, 1965
Malus sp.	Smit & Wieringa, 1953
Picea sitchensis (Bong.) Carr.	Hayes, 1965
Pinus nigra Arn. var. austriaca Asch. & Gr.	Gremmen, 1959
Pinus nigra Arn. var. corsicana Schn.	Gremmen, 1959; Batko et al., 1958
Pinus sylvestris L.	Kendrick & Burgess, 1962; Hayes, 1965
Prunus persica Batsch	Smit & Wieringa, 1953
Quercus sp.	Smit & Wieringa, 1953

living leaves or only on senescent leaves which had not yet fallen. There are, however, some reports listed in Table 41 which do indicate that fungi have been isolated from living tree leaves. A. pullulans was isolated from living leaves of beech and amelanchier (Smit & Wieringa, 1953), P. sylvestris (Batko et al., 1958), and ash, birch, hazel and oak (Hering, 1965).

Studies of the phylloplane by Ruinen (1956, 1961) and Last (1955a) created an awareness of the possible importance of saprophytic micro-organisms on the surfaces of living leaves, and this is reflected in later work done on tree leaf saprophytes. Hering (1965) carried out a more detailed examination of fungi on living green leaves of several tree species (Table 42). A detailed account of fungi on living beech leaves followed by a sequential study on the resulting leaf litter was given by Hogg & Hudson (1966). Friend (1965) surveyed the sooty moulds on lime leaves.

Bacteria on tree leaves have been studied by Crosse (1959) who found Pseudomonas mors prunorum (usually considered to be pathogenic) living epiphytically on leaves of cherry trees. Jensen (1971) has observed bacteria on beech leaves and litter over four seasons. Studies on the whole microflora, fungi, yeasts and bacteria, have been done for apple (Hislop & Cox, 1969), Douglas fir (McBride, 1969) and larch leaves (McBride, 1970).

The whole study of micro-organism populations of plant buds including those of perennials has been reviewed by Leben (1971). Later work on the buds of apple, cottonwood, and white pine (Leben, 1972) has shown that few bacteria were found in the buds of the two deciduous trees confirming the observations of Keener (1950, 1951)

Table 42. Tree species from whose leaves Aureobasidium pullulans has been isolated

Tree species	Reference
Acer pseudoplatanus L.	Pugh & Buckley, 1971(a)
Amelanchier sp.	Smit & Wieringa, 1953
Corylus avellana Ehrh.	Hering, 1965
Fagus sylvatica L.	Smit & Wieringa, 1953; Hogg & Hudson, 1966
Fraxinus sp.	Hering, 1965
Larix decidua Mill.	McBride, 1970
Malus sp.	Hamilton, 1959; Hislop & Cox, 1969
Pinus sylvestris L.	Batko et al., 1958; Kendrick & Burges:, 1962
Populus trichocarpa Torrey & Gray	Bier, 1965
Pseudotsuga menziesii (Mirb.) Franco	McBride, 1969
Quercus sp.	Hering, 1965
Salix babylonica L.	Lamb & Brown, 1970
Tilia sp.	Friend, 1965

and only a few more were found consistently in the buds of white pine. Low numbers of yeasts and fungi, a few hundred per gram, were associated with these tree buds. Warren (personal communication) surveyed the bud microflora of seven deciduous tree species and concluded that these could be grouped into species such as lime and ash, in which the inner bud scales and furled leaves supported microbial growth, and others, sycamore, chestnut, beech, oak and elm in which the micro-organisms were usually restricted to the outer bud scales. This confirms the earlier work of Pugh & Buckley (1971b) in which they found A. pullulans restricted to the outer bud scales of sycamore. Warren (personal communication) also found that 80% of the micro-organisms which he isolated from tree buds were white yeasts, pink yeasts, A. pullulans and Cladosporium sp.

The majority of work in this field has been concerned with a general survey of the phylloplane microflora. However, some work has been directed at specific organisms or groups of organisms excluding studies on pathogens and disease. Jones (1970) worked on nitrogen fixing bacteria associated with needles of Douglas fir and Last (1970) isolated S. roseus from many tree species.

From these studies on the phylloplane microflora of trees, it has emerged that a large variety of micro-organisms occur on the surface of leaves and that some of these organisms were ubiquitous. One of the most common fungi was A. pullulans which has been isolated from the leaves of many trees (Table 42). Cladosporium spp.have also been isolated from leaves of many different trees. The isolation of one type of organism from many species of plant is not restricted to filamentous fungi as the pink yeast S. roseus has been isolated from

rowan (Last in Last & Deighton, 1965), oak, horse-chestnut, elm and apple (Last, 1970), Douglas fir (McBride, 1969), European larch (McBride, 1970) and sycamore (Pugh & Buckley, 1971a). Not all abundant phylloplane micro-organisms have a multispecies habitat. Trimmatostroma sp. was found in high numbers only on needles of Scots pine (Kendrick, 1957). Similarly Protostegia eucalypti was only found on E. regnans (Macauley & Thrower, 1966).

Saprophytic phylloplane micro-organisms have not been studied in isolation. Investigations have also been done on the interactions of these saprophytes with leaf pathogens or pathogens which enter through leaves or leaf scars. The literature on these interactions has been reviewed above (p.123).

(b) CHRYSOMYXA ABIETIS: A RUST DISEASE OF NORWAY SPRUCE NEEDLES

Chrysomyxa abietis, a needle rust of Picea abies Karsten was first described in 1840 (Unger, 1840). An even earlier description of this fungus is available under the name of Blenoris abietis (Wallrohf, 1834). Thus some confusion exists as to the authority to whom this fungus should be attributed. Later papers quote a variety of authorities including Unger, Wallrohf, Winters and Nuger. Winters chief taxonomic works were written much later between 1881 and 1887 in Rabenhorst's Kryptogamic Flora (1884-1932). Nothing can be found about Nuger although it may be more than coincidence that both the names Unger and Nuger contain the same letters and thus it may be possible that a printer's error occurred. Wilson and Henderson (1966) credit Unger as the correct authority and as such it will be accepted in the present study.

Since the works of the early nineteenth century mentioned above, most of the information available from the literature is in the form of reports on the occurrence of C. abietis especially when outbreaks of the disease have been severe. Silvio (1896) reported an attack from the Haute Savoie. Delforge (1908) noted an outbreak of the disease in Luxembourg and also gave a full description of the life history and some conditions which appear to favour successful infection. The first record of C. abietis in the United Kingdom was in 1911 (Somerville, 1911) from Durris forest near Aberdeen, but the same author stated (Somerville, 1915) that he had received no further records of its occurrence. In the same year C. abietis was stated to have recently made an appearance in northern Scotland and to be widely spread in Europe especially in Switzerland and Germany (Borthwick & Wilson, 1915). This was followed by reports from Luxembourg (Delforge, 1930), and Switzerland (E.B., 1933) in which an epidemic of this rust disease was described. Wilson (1934) found C. abietis, near Kelso on P. sitchensis growing in the vicinity of infected P. abies. Jørstad (1940) considered C. abietis to be the most important rust species infecting trees in northern Norway after observing particularly virulent outbreaks between 1936 and 1941 during which Picea pungens Engel. and Picea engelmanni (Parry) Engelmann were also infected (Jørstad & Roll-Hansen, 1943). Boyce (1943) noted that C. abietis had been introduced into North America on nursery stock but that no spread had been observed. It is therefore surprising that Savile (1950, 1955) makes no mention of C. abietis in his list of the Chrysomyxa species in North America.

In 1951 there was a severe outbreak of C. abietis in Scotland which was described by Murray (1953). He correlated the severity of this outbreak with the unusually cold spring which may have delayed the flushing of new needles of Norway spruce (Murray, 1953, 1955). This point was further elaborated by Peace (1956), who stated that successful infection depended on a delicate balance between fruiting of the fungus and the flushing of new needles of spruce which is seldom achieved in the U.K. and so serious attacks by C. abietis are comparatively rare. Even so, later reports of C. abietis were received for east and south Scotland, north and south west England and south Wales (Phillips, 1964). In the colder regions of Europe the outbreaks of C. abietis may be severe and occur more frequently. In Lithuania virtually all mixed stands of spruce and deciduous trees are infected (Mikalaikevicius & Rimkus, 1963) and in the Archangel region C. abietis is endemic (Drackou, 1965).

Apart from the reports on the occurrence of C. abietis little work has been done on the fungus itself. Lindfors (1924) gave a very complete account of the development of the teleutosorus. Delforge (1908, 1930) and Borthwick and Wilson (1934) described the life history of C. abietis while Wilson (1934) noted that the sorus was infected frequently by the hyper-parasite Darluca filum (Biv-Bera. ex Fr.) Cast. Apart from the weather, site conditions, high soil moisture and high stand density may favour infection by C. abietis (Delforge, 1908, 1930; Drackou, 1965). The severe defoliation caused by C. abietis attack can result in a pronounced loss in height increment of the spruce trees, especially in plants 0.5 to 1 m tall (Drackou, 1965). This is the present state of knowledge concerning the incidence and development of C. abietis and

its effects on the host trees.

(c) RHYTISMA ACERINUM: A PATHOGEN OF ACER SPECIES

The early investigations on R. acerinum were more or less confined to the study of different biologic forms of this parasite. Julius Müller (1893) attempted to use morphological parameters to show that several species were embraced by the title R. acerinum. He discovered gradual variations which he classified into four types according to the relative diameter of the stromata. Karl Müller (1912a) studied the problem of variation in biological forms by inoculating different Acer spp. with ascospores derived from different acer hosts. He used Acer pseudoplatanus L., Acer platanoides L. and Acer campestre L. and found that R. acerinum isolated from A. platanoides could infect A. campestre but that the R. acerinum from A. pseudoplatanus could not infect A. campestre very easily but could infect A. platanoides. Further he found that R. acerinum from A. campestre could infect A. platanoides but not A. pseudoplatanus. The fungus on sycamore was referred to as Rhytisma pseudoplatanus Müller, but this distinction has not been consistently maintained and most records of R. acerinum in U.K. could be referred to R. pseudoplatanus Sensu Müller although the disease may be found on A. campestre (Jones, 1944).

Accounts of the life history of R. acerinum have been given by Massée (1901), Müller (1912a, 1912b) and Jones (1923, 1925). The detailed studies of Müller and Jones include considerable information concerning ascospore release. The cytology of sorus development of R. acerinum was well described by Jones (1925), supported by the later work of Aragno (1968) who used the Feulgen staining technique to

confirm some of the observations made by Jones as well as to clarify some essential details. The attempted culture of R. acerinum on artificial media was described by Schweizer (1932) who pointed out that stromata must overwinter in moist conditions to remain viable. Although Schweizer managed to culture R. acerinum, he was not able to achieve successful reinfection of Acer spp. with this isolate. The infection process has been described by Müller (1912a), Bracher (1924) and Jones (1925). Müller (1912a) showed that ascospore discharge from the stromata was favoured by the alternation of damp and dry weather. These three authors disagree on the possible entry points into host tissue essential for successful infection. Both Müller and Bracker found that if the stromata was pushed against the upper surface of the leaf then cell damage or cuticular damage may result but not infection, which, however, could occur via the stomata in the lower epidermis of the leaf. Having observed, in spore discharge experiments, that the majority of ascospores land on the upper surface of the leaf, Jones (1925) speculated on the possibilities of cuticular penetration although he was unable to verify this point. The length of time between inoculation and the appearance of the familiar yellow patches on leaves, which precede the eruption of the black stromata, varied considerably in different years according to seasonal variations in moisture and temperature (Jones, 1925).

This marked dependence on climate for survival, infection, and development might be expected to limit the spread of R. acerinum. Maxwell (1933a) found R. acerinum-free plantations in Corrour in Inverness-shire. These plantations were 30 years old at an elevation of 366-427 m on a previously unplanted site. He

suggested that the severe climate, height and distance from other woodlands might prevent the occurrence of the disease, but later that same year Maxwell (1933b) noted that a few trees were infected by R. acerinum. Batko (in Peace, 1962) noted the absence of R. acerinum from another remote area, the Isle of Skye. Peace (1962) observed that R. acerinum is often absent from areas of heavy atmospheric pollution in and around large industrial cities, so it would seem that this atmospheric pollution may be acting as a control. Other control methods have been attempted. Passavalli (1933) found that a 1% solution of Bordeaux mixture was effective when sprayed on young leaves and that other copper fungitoxic solutions applied to the leaf litter prevented stromatal development. He also gave a list of supposedly resistant Acer species, although he did not conduct infection experiments on these trees.

On a field scale, the expense of applying fungitoxic substances could well be prohibitive and in cities amenity trees may well be protected by atmospheric pollution, so that it is arguable whether control measures are worthwhile. Little is known of the effects of R. acerinum on Acer spp. Massée (1901) stated that R. acerinum infection caused premature defoliation and thus the wood was imperfectly matured and so highly susceptible to other parasitic fungi, especially Nectria cinnabarina (Tode) Fr. Young trees soon succumb to the combined attacks of these fungi and in some cases branches of larger trees can be killed. Peace (1962) observed that apart from nurseries the only reason for control would appear to be the disfiguring appearance of the disease during late summer on sycamores or maples planted for ornament. This last

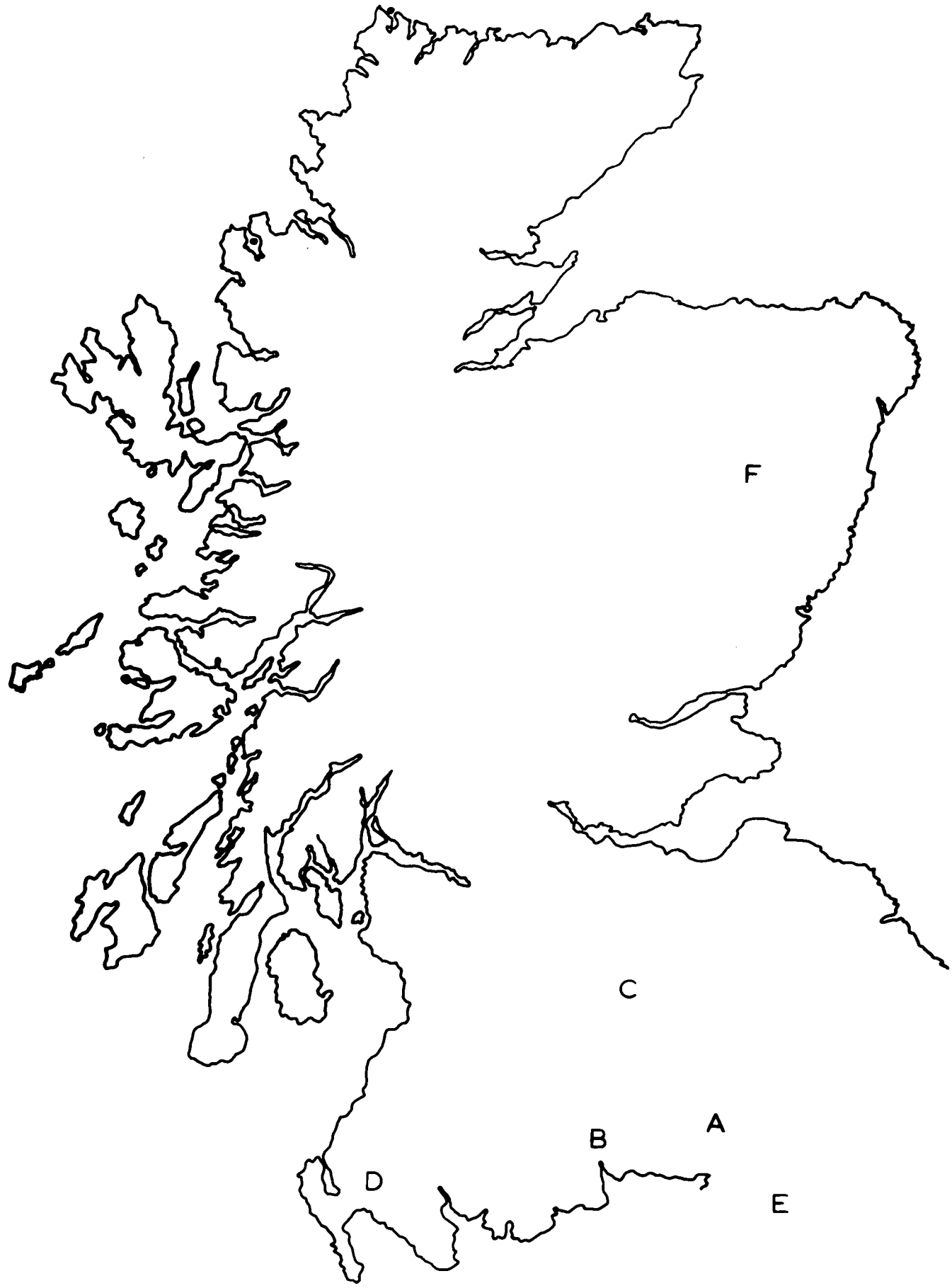
point may well be important in the future in view of the increasing use of trees for amenity value coupled with the proposed controls on atmospheric pollution. The presence of 'Tar spots' and premature leaf fall might well preclude the planting of an otherwise hardy, easily grown, pleasant deciduous shade tree, whose timber may also be used for veneer.

SITE DESCRIPTION

In late autumn 1970, some forests and woodlands in the Edinburgh area were surveyed for the presence of C. abietis on P. abies and R. acerinum on A. pseudoplatanus. Practically no C. abietis was observed and so the survey area was extended to include forests which were within 150 Km of Edinburgh.

The nearest forest showing a moderate level of infected trees was Castle Ø'er forest Dumfriesshire (G.R. NY211950) about 125 Km south west of Edinburgh (Fig. 49). Compartment 137 of this forest was chosen as the sampling site as it contained about equal proportions of C. abietis infected and uninfected trees of the same age. These trees had been planted in 1950 and prior to that time the land had been used for sheep farming. The aspect of the site was south west at a height of 259 m. The soil was a deep peat which had been ploughed. Meteorological data for this site was obtained from an observatory at Eskdalemuir approximately 6.5 Km away.

In the case of R. acerinum a suitable site was found close to Edinburgh at Newbattle Abbey, Midlothian (G.R. NT334657). In the abbey grounds sycamore trees planted in 1951 were growing in a mixed stand with Norway maple. Both of these Acer spp. had been infected by R. acerinum. The site sloped slightly to the west at a height of 54 m. The soil was a brown earth which had been continuously planted with hard wood trees for at least 100 years. Meteorological data for this site was obtained from an observatory at Langhill Farm approximately 6 Km distant.



A. WAUCHOPE FOREST, ROXBURGH.
B. CASTLE O'ER, DUMFRIESHIRE.
C. CAMPS RESERVOIR, LANARKSHIRE.
D. GLENTROOL FOREST, AYRSHIRE.
E. WARK FOREST, NORTHUMBERLAND
F. DRUMTOCHTY FOREST, ABERDEENSHIRE.

Fig. 49. Incidence of C. abietis within forests in southern Scotland and northern England (1970-71)

PRELIMINARY INVESTIGATIONS

In Castle O'er forest *C. abietis* infected and uninfected trees were found close together within one compartment. During March 1971 all infected trees within this compartment were examined to ascertain whether the trees which were already infected by *C. abietis* were more likely to be infected in subsequent years than trees which were previously uninfected. This involved the examination of all trees infected by *C. abietis* in 1970 in order to ascertain whether there was an obvious loss of needles in the previous year. Any needles from 1969 remaining on these trees were examined for sign of *C. abietis* infection. The observations on these trees are given in the table.

Table 43. Extent of predisposition to infection in 1970 by *C. abietis* of *P. abies* trees infected in 1969

	1969	1970
Infection only in 1969	7	-
Infection in both years	35	35
New infection in 1970	-	6

83.3% (± 11.2%) of the trees infected in 1970 had been infected by *C. abietis* the previous year. This result is highly significant ($p = .001$) and demonstrates that trees infected one year are more likely to be infected in the following year. It should be remembered that this analysis takes no account of the effects of suboptimal weather conditions in any one year which are also thought to be

important (p.209). Observations would have to be taken over several years to obtain really accurate data, but this analysis does give an indication of some effect. This survey indicated that it might be worthwhile to sample new needles from trees both infected and uninfected by C. abietis in the previous year to ascertain whether the early stages of this disease produce any changes in the saprophytic phylloplane microflora.

In the case of R. acerinum easily visible signs of infection do not appear until August (Massée, 1901). Thus it is nearly impossible to show that infection has taken place earlier in the season (Jones, 1925). Therefore only later in the season was it practical to obtain samples to investigate the phylloplane microflora of infected leaves.

METHODS

ISOLATION AND ENUMERATION OF SAPROPHYTIC PHYLLOPLANE MICRO-ORGANISMS

The problems involved in the isolation of micro-organisms from the leaf surface and the range of techniques available for use in these studies have been discussed previously (p.27). Only preliminary experiments on the relative efficiency of washing and maceration techniques will be mentioned here, followed by the procedures adopted in this study of the micro-organisms on the leaves of two tree species.

The relative efficiency of washing and maceration techniques for removing micro-organisms from the needles of Norway spruce was tested by the following method. Three separate samples of spruce needles

were collected. Suspensions of needles were prepared using a homogeniser (Jonke & Kunkel ultra-turax) for one minute. The resulting suspension was diluted and cultured. Leaf washings were prepared by vigorously shaking needles in water on a wrist action shaker (Griffin & George) for one hour. These washings were diluted and cultured. A parallel experiment was conducted using 0.01% Tween 80 solution instead of water for homogenising and washing. The results obtained for the isolation of bacteria are given (Table 44).

Plate counts of the suspensions formed by homogenisation were slightly higher than those prepared by shaking. The use of dilute Tween solution resulted in more consistent plate counts rather than an overall numerical difference. This may be the result of breaking up colonies or clumps of bacterial cells. Similar results were obtained for the washing and maceration of sycamore leaf discs.

Two broad spectrum media were used throughout the sampling period, one for bacteria and the other for filamentous fungi and yeasts. The media which supported the highest numbers of organisms were chosen af after an initial trial. The medium for filamentous fungi and yeasts was MEC (p. 32) and for bacteria PYN (p. 32).

The inoculum may be applied to plates by either surface plating as used previously (p. 34), or by pour plating. In this technique the inoculum is pipetted into an empty petri dish and a set volume of molten agar medium (at about 45^{o}C) added, mixed well by agitating and allowed to set. This latter method, while being less convenient, does permit the use of larger aliquots of inoculating suspension to ensure that sufficient colonies grow on a plate to allow a meaningful count to be made (Montegut, 1960; Jensen, 1968). It was necessary to use

Table 44 Comparison of numbers of bacteria isolated from Norway spruce needles using washing and maceration techniques with and without Tween 80

Suspension	Water			0.01% Tween 80		
1						
Washed	857	623	745	750	762	754
Macerated	1000	847	738	905	894	912
Washed then macerated	17	20	17	20	25	20
2						
Washed	453	526	472	450	440	459
Macerated	400	525	432	462	471	466
Washed then macerated	10	5	5	10	10	10
3						
Washed	1000	840	950	980	1000	1050
Macerated	1100	990	1020	1010	1020	1000
Washed then macerated	20	30	20	25	25	30

this technique for the isolations from Norway spruce.

Techniques used for isolating micro-organisms from both Norway spruce and sycamore were similar and so the full procedure is given for isolations from Norway spruce followed by a short section outlining the differences in technique used for the isolations from sycamore.

(1) SAMPLING PROCEDURE

Samples were taken from Norway spruce trees in compartment 137 in Castle O'er forest. Trees were selected in which the spring flush of new needles occurred within the same week. Three samples were taken from trees infected by C. abietis in the previous year and three samples from trees free from C. abietis infection. Samples were removed from the trees using alcohol flamed secateurs and forceps, placed in new polythene bags and enclosed in a cool polystyrene box for transport back to the laboratory. Needles were removed from the cut branches using sterile scalpel and forceps, which were flamed between samples, and placed in sterile boiling tubes. Approximately 2 g of needles were used for each sample which was weighed to the nearest 0.1 g. 10 ml of 0.01% Tween 80 solution were added to each sample which was macerated by the homogeniser for 60 seconds. The blades of the homogeniser were sterilised by flaming with alcohol before the maceration of each sample. A dilution series was prepared from the resulting suspension and 1 ml aliquots were pipetted into six empty sterile petri dishes for each dilution. 15 ml of MEC agar was added to three dishes and 15 ml PYN agar added to each of the remaining dishes. All dishes were agitated to mix the inoculum with the culture medium which was then allowed to set before incubating in an inverted position for six days at 20^{o}C before counting the colonies

using a Gallenkamp colony counter. Only plates containing between 30 and 300 colonies on PYN agar (Jensen, 1968) and less than 50 colonies on MEC medium were counted (Montegut, 1960). The number of micro-organisms present on the leaf surface was calculated on the basis of number per gram fresh weight.

The procedure adopted for the isolation of micro-organisms from sycamore leaves was essentially the same as that given above, but there were some variations. Five leaves were collected from each of four trees, placed in clean polythene bags and brought back to the laboratory. Four 10 mm discs were removed from each of the five leaves in a sample using a sterile cork borer. The samples of 20 leaf discs were macerated in the same manner as that described above. The resulting suspension was used to make up a dilution series. Aliquots (0.1 ml) of the resulting suspensions were pipetted onto the surface of three plates of MEC agar and three plates of PYN agar at each dilution. Inoculum was spread over the surface of the medium using a sterile glass spreader. Culture plates were incubated and counted in the same way as that described above. Numbers of micro-organisms isolated was expressed in terms of numbers cm^{-2}.

(2) IDENTIFICATION OF PHYLLOPLANE MICRO-ORGANISMS

Qualitative analysis of the microbial populations was done using the same proportional sub sampling procedure described previously (p.34).

PATHOGEN SPORE GERMINATION AND INFECTION TRIALS

1) C. ABIETIS

a) Isolation of sporidia and teleutospores

Two methods were used to obtain sporidia and teleutospores. First, sporidia and teleutospores were removed directly from ripe teleutosori using a sterile needle or paintbrush. The second method used spruce needles bearing swollen but unruptured teleutosori. These needles were placed directly over a slot cut in a piece of sterile muslin so that the needles were supported by both ends. This piece of muslin was stretched across a petri dish fitted as a moist chamber (Costonis et al., 1970) and having slides or agar films in its base to catch the spores falling after the teleutosori had ripened and ruptured.

b) Spore germination experiments

Sporidia and teleutospores collected in the ways outlined above were placed either in droplets of sterile deionised water or thin films of 0.75% water agar (Difco), supported on slides or coverslips. The slides were placed in moist chambers at temperatures from 5°C to 25°C and examined for germination at different time intervals using the criteria of Manners (1949). The coverslips were placed in humidity cells (p.63). Another series of tests was set up using dilute solutions of amino acids or spruce leaf extract instead of deionised water. Cysteine, Ascorbic acid and citric acid at concentrations of 10^4 - 10^5 ppm were used which had been shown to stimulate germination in North American Chrysomyxa spp. (Gould, 1964). All spore suspensions were observed using dark field phase contrast microscopy.

c) Infection experiments

Three methods were used in infection experiments:

1) Sporidia and teleutospores collected by the methods described above were used in water suspensions which were sprayed onto newly flushed needles of young Norway spruce trees growing in a plot at King's Buildings, University of Edinburgh. Some of the sprayed trees were kept in moist conditions by placing a cloche over each tree.

2) Newly flushed needles of young spruce trees were inoculated directly by rubbing ripe open teleutosori over the young needles. Again some trees were placed under polythene cloches.

3) Needles of Norway spruce bearing ripe teleutosori of C. abietis were suspended over newly flushed needles of young Norway spruce trees. The method was essentially that of Costonis, Sinclair and Zycha (1970) except that a pad of moist cotton wool was placed above the detached needles to prevent drying out. The trees and suspended infected needles were enclosed within polythene cloches.

d) Enumeration of Sporidia of C. abietis in the airspora

Sporidia of C. abietis are released in the spring. These bodies alight and may infect needles of Norway spruce. It is thought that these sporidia are probably distributed through the air. No work seems to have been done either to determine the possible pattern of release or to confirm that they are airborne. A Burkhard volumetric spore trap was used to find out if the sporidia were airborne and to determine any possible pattern of release. This spore trap was placed on a dexion framework so that the orifice was 1.5 m above ground level, and positioned in a small clearing (approximately 5 m square) among 21 year old Norway spruce trees some of which were infected by

C. abietis. The spore trap operated by sucking in air at a known rate through a very precisely cut orifice. Immediately behind this orifice was the edge of a wheel bearing sticky coated melinex tape (adhesive used: Vaseline in 10% paraffin wax in toluene) upon which particles in the air drawn into the machine impact. This wheel revolved once per week. Each week the tape was changed. The spore trap was run continuously throughout May and June which included the period of new needle flushing for Norway spruce in Castle O'er forest. It is at this time that teleutospores develop and release sporidia which may infect new spruce needles (Delforge, 1908). The used tapes were examined by cutting the tape into 48 mm lengths representing a 24 hour period. These pieces of tape were mounted on microscope slides and covered by a 22 x 50 mm coverslip. The resulting slides were examined (x 650) and the number of sporidia occurring in an 8 mm length of tape (= 4 hour period) counted.

2) R. ACERINUM

a) Isolation of Ascospores

Fallen sycamore leaves bearing stromata were collected in autumn and stored overwinter in moist conditions at below $5^{o}C$. Further collections of infected leaves were made in spring from moist areas in the litter layer. The surface sterilisation of some stromata was tried using either mercuric chloride (1% solution) or sodium hypochlorite (1% available chlorine). Areas of the leaf bearing stromata were dissected out and placed in petri dishes kept moist by placing damp filter papers in the lids. These dishes were placed at room temperature in a darkened room. When the stromata had ruptured the lid of the dish was removed and a horizontal beam of light shone across the dish. Any ascospores being forcibly ejected should be visible in

the light beam (Jones, 1925). The ascospores were collected either by means of dragging a small sterile wire loop along the fracture line in the stromata or by means of placing coverslips beneath stromata, held in the lids of petri dishes by Vaseline, to catch the ejected ascospores.

b) Spore germination experiments

Ascospores collected by the methods described above were placed in cells (p. 61) and subjected to different conditions of humidity, temperature and light for various time intervals before examination by dark field phase contrast microscopy. Inoculation onto thin films of prune agar was also tried (Jones, 1925).

c) Infection experiments

Attempts were made to infect young leaves on young sycamore trees both by spraying the trees with water suspensions of ascospores and by direct wound infection using a needle loaded with ascospores to wound the leaf surface. Some of the trees treated by these methods were kept in high humidity conditions by use of polythene cloches.

d) Axenic culture

Ascospore suspensions were used to inoculate prune agar used by Jones (1925) in germination experiments and Schweizer's medium (Schweizer, 1932), which was then incubated at a range of temperatures from 10-25^{o}C.

RESULTS

P. ABIES/C. ABIETIS

1) ISOLATION AND ENUMERATION OF THE SAPROPHYTIC PHYLLOPLANE MICROFLORA

a) Climatic data

These data were obtained from Eskdalemuir Observatory, Dumfries-shire.

i) Temperature

Monthly mean values for maximum and minimum temperature (Fig. 50) show that the maximum temperature rose sharply during May and July to give a peak summer temperature of 18.4°C, while the minimum temperature rose in a linear manner at a rate of 2.6°C per month from April until the end of July before stabilising and then declining.

ii) Rainfall

Total rainfall per fortnight (Fig. 50) in relation to the sample collection date shows that several samples were taken after dry periods, notably samples for April, June and July. An examination of the hourly rainfall records revealed that no rain had fallen in the week prior to June 15. Some rain had fallen within the 24 hour period prior to the collection of all other samples.

iii) Humidity

The relative humidity at Eskdalemuir had been recorded at hourly intervals. These records were processed as previously (p. 36). Figure 50 shows that in general high humidity occurred for about 47% of the time. Exceptions were the first fortnight in June (22% of the

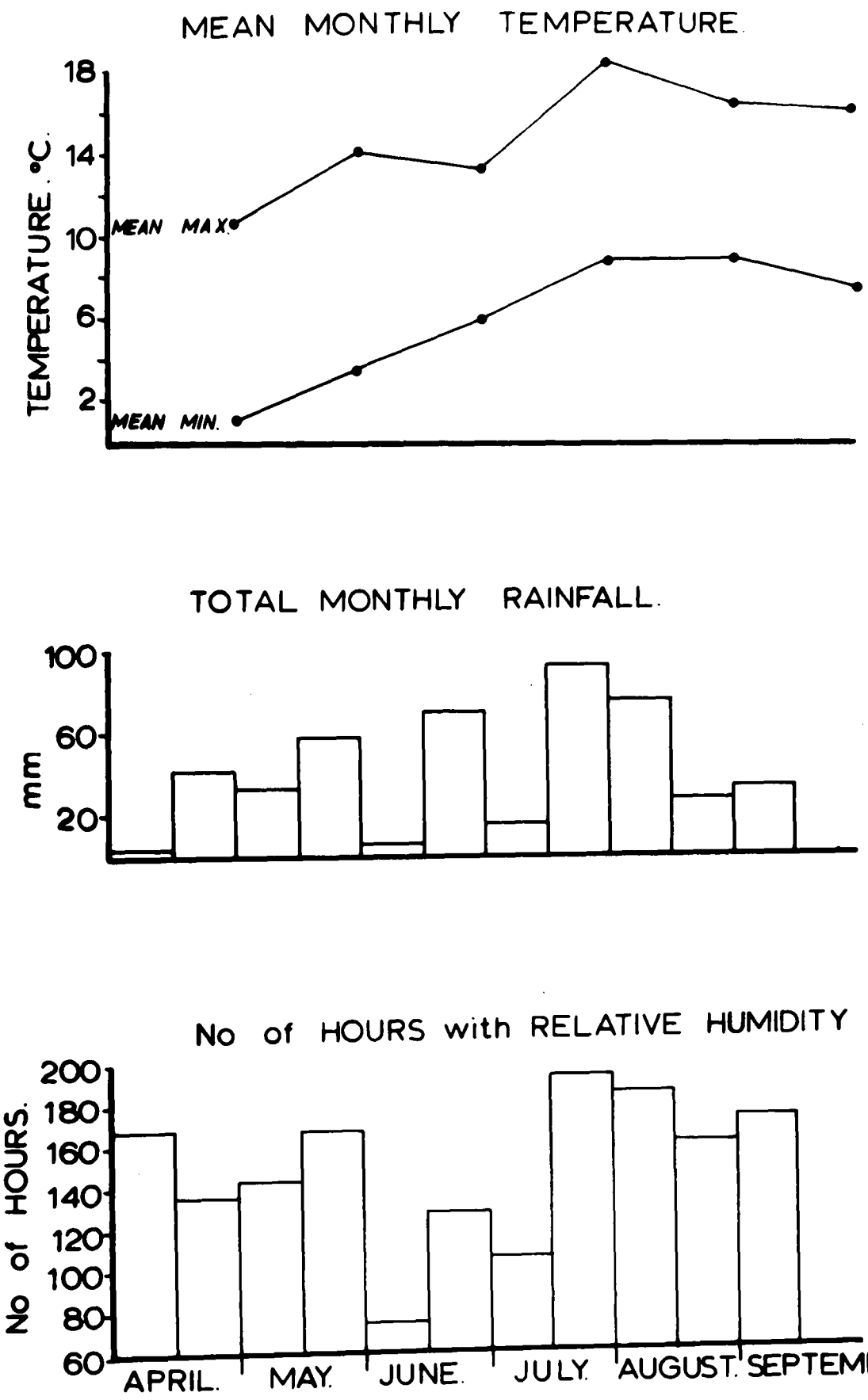


Fig. 50 Meteorological data April to September 1971
Eskdalemuir Observatory

time) which was dry, and to a lesser extent the first fortnight in July (30% of the time period).

b) Enumeration of the saprophytic phylloplane microflora

In the autumn of 1971 all trees from which needles had been taken for use in the survey, were examined for symptoms of infection by C. abietis. All trees which had been infected in 1970 were found to have new needles infected in this present year. None of the other trees from which samples had been taken were found to be infected.

Numbers of filamentous fungi, yeasts and bacteria isolated from the needles of P. abies during the period April to September 1971 are given in Figs. 51,52,53, respectively. Two sets of values are given for the April samples for both infected and uninfected trees. The points surrounded by the box were obtained by macerating the whole bud, whereas the other set of data for 15 April, 1971, was obtained by maceration of bud material after the removal of the outer bud scales.

i) Filamentous fungi

Although considerable numbers of fungal propagules were isolated from outer bud scales, very few were found within the bud. With the onset of needle flushing there was a sharp increase in numbers of fungi on the new needles themselves (Fig. 51) . Isolation later in the season showed little change from the value attained in May although there was a slight increase in fungal numbers during September. Although the mean values for numbers of fungi show that isolation from needles infected by C. abietis were consistently lower than those from uninfected needles, it was only in the May sample that this difference was significant ($p = .05$).

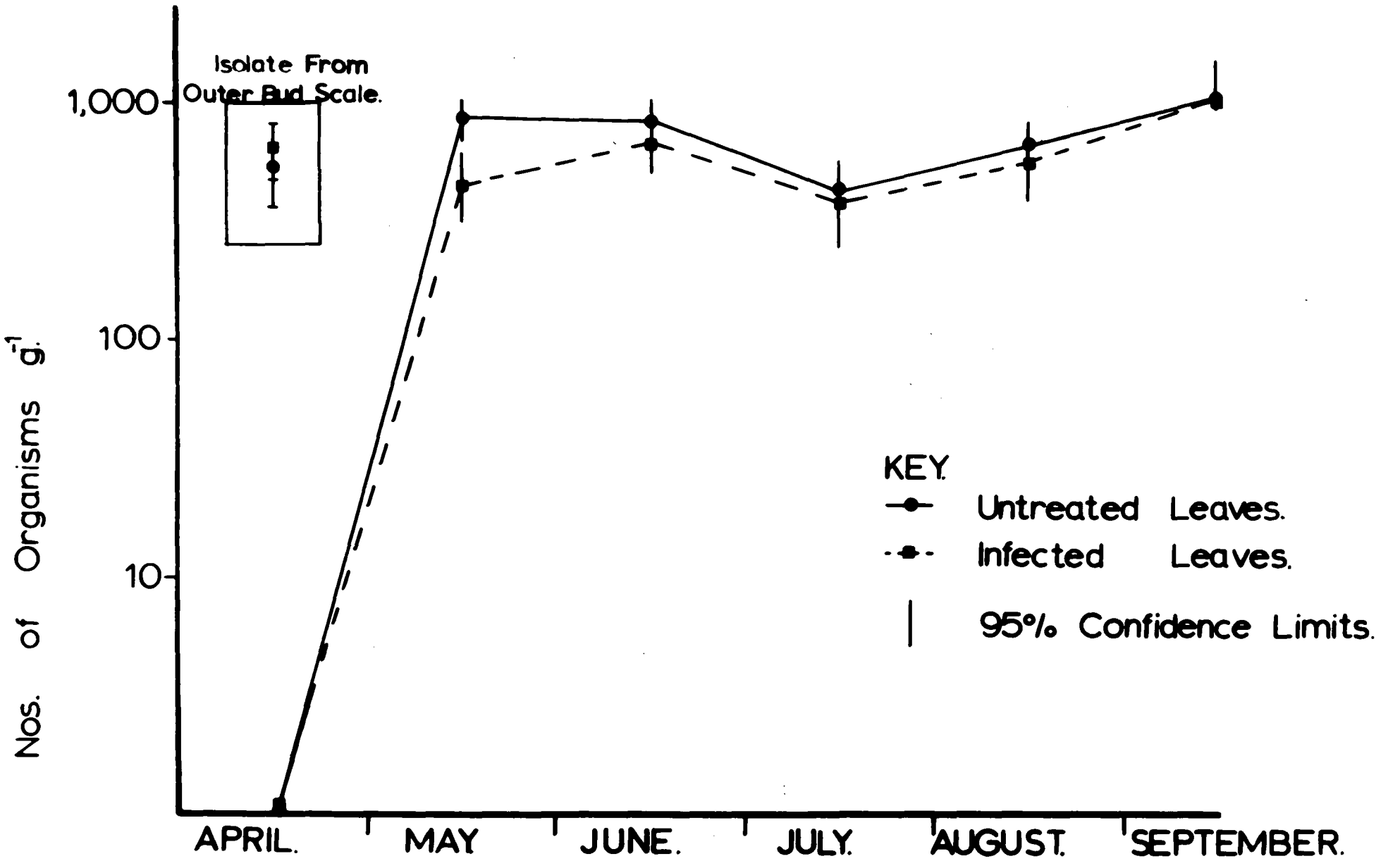


Fig. 51 Norway spruce: Numbers of filamentous fungi isolated from needles in 1971

ii) Yeasts

Yeast cells were isolated from within the unopened bud, but the numbers were so low that to represent this value as an accurate estimate would be unrealistic and so they were recorded here only as a presence. Large numbers of yeast cells were isolated from the outer bud scales. In May numbers of yeast cells isolated were significantly higher ($p = .05$) than numbers isolated later in the year (Fig.52). It was only in this (May) sample that a significant difference ($p = .05$) occurred in the numbers of yeasts isolated from infected and uninfected trees. In all other samples the difference in numbers was not significant, although the mean number of yeast cells isolated was consistently higher for trees infected by C. abietis.

iii) Bacteria

Numbers of bacteria isolated were considerably higher than numbers of micro-organisms in the other two groups. Bacteria were isolated from within the unopened bud in April. After an initial peak in May the bacterial numbers slumped during June (Fig.53), but increased later in the year. It was only in May and September that there was a significant difference in bacterial numbers isolated from C. abietis infected trees and uninfected trees. The estimates of bacterial numbers for C. abietis infected trees for the month of August are missing owing to the contamination of a particular batch of culture media.

c) Identification of phylloplane micro-organisms

The results presented here are concerned with the micro-organisms isolated most frequently or in large numbers. A full list of all the species of filamentous fungi isolated appears in Appendix 2.

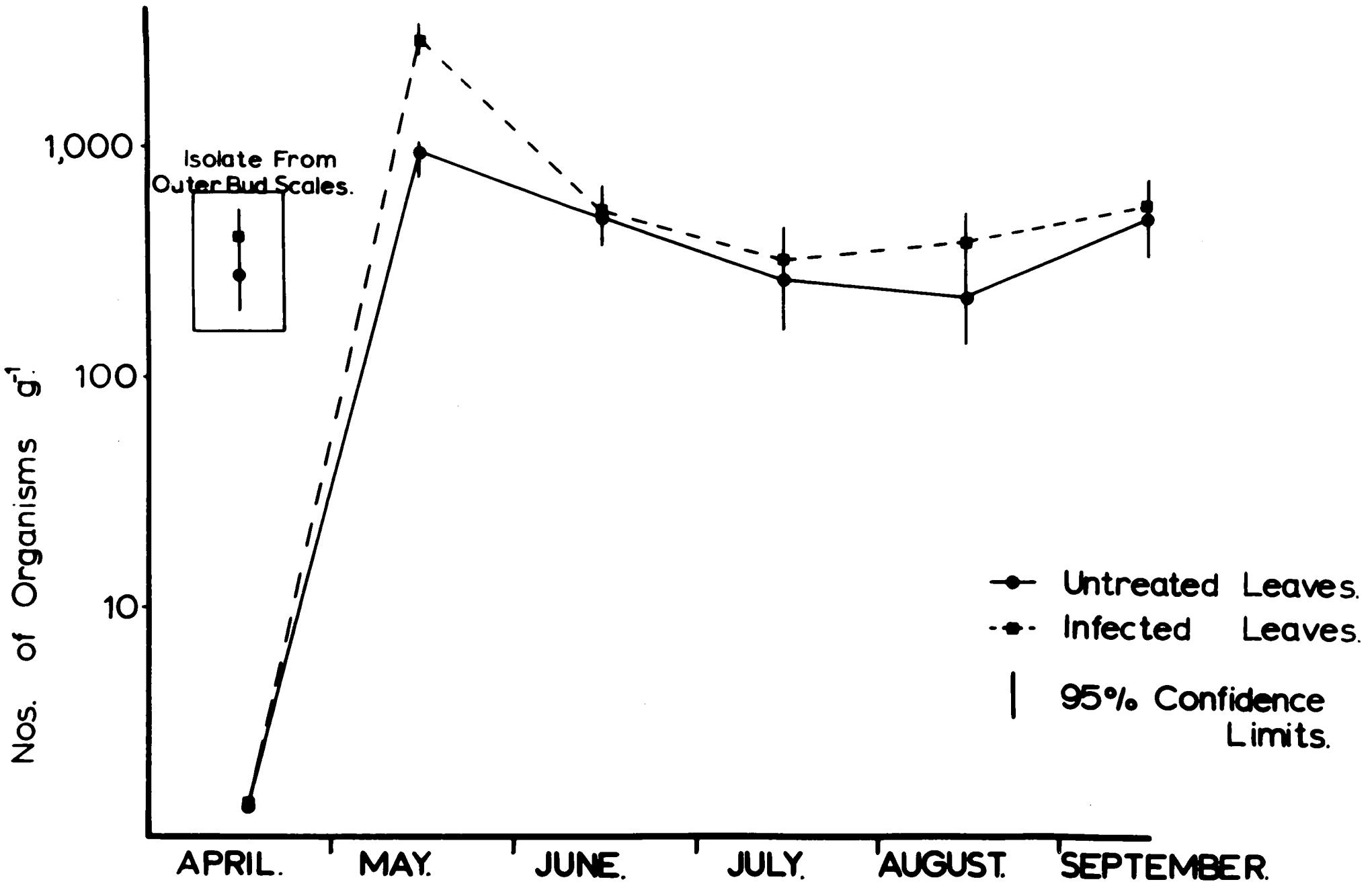


Fig. 52 Norway spruce: Numbers of yeasts isolated from needles in 1971

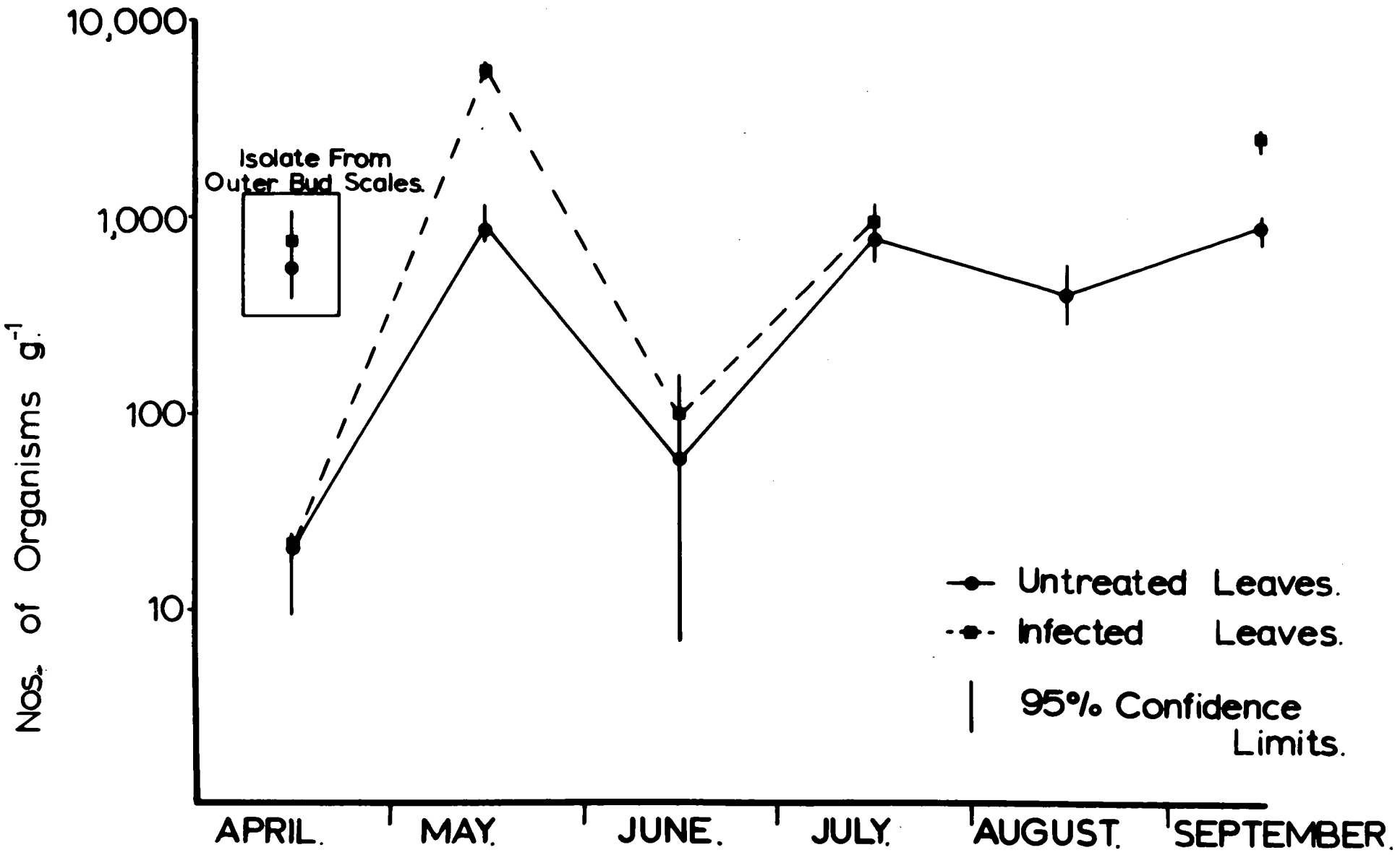


Fig. 53 Norway spruce: Numbers of bacteria isolated from needles in 1971

Table 45. Norway Spruce: Species of filamentous fungi isolated from needles in the greatest numbers at each sampling date.
(Relative numbers g^{-1} fresh weight calculated from proportional sub-sampling data: % of total filamentous fungi given in parentheses).

Sample	April	May	June	July	August	September
isolate from healthy needles	Pre-flushing	Post-flushing				
	Penicillium funiculosum Thom	Cladosporium herbarum (Pers.)Link ex S.F. Gray	C.herbarum	Fusarium oxysporum schlect.	Mucor genevensis Lend.	Humicola sp . 206 (16.8) C. herbarum
	456 (88)	200 (23) Heptaster sp. 200 (23)	476 (57.2)	496 (76.7)	496 (76.7)	137 (11.3) Cladosporium cladosporioides (Fresen.) de Vries 140 (11.5) C.herbarum
isolate from C.abieties infected needles	P.funiculosum	Aspergillus fumigatus Fresen.	Penicillium cyclopium West.	white sterile mycelium	C.herbarum	
	517 (80.4)	212 (46)	263 (38.9)	128 (34)	150 (27.3) C.cladosporioides 163 (29.6)	622 (53)

April figures refer to isolates from outer bud scales only.

Table 46. Norway spruce: Changing numbers of species of filamentous fungi isolated from needles in several monthly samples.
(Relative numbers g^{-1} fresh weight calculated from proportional sub-sampling data:% of total filamentous fungi given in parentheses).

Isolate from healthy needles	April	May	June	July	August	September
	Pre-flushing	Post-flushing				
Cladosporium herbarum (Pers.)Link ex S.F. Gray	11 (2.2)	200 (23)	476 (57.2)	13 (3.0)	-	137 (11.3)
Cephalosporium sp.		174 (20)	96 (11.5)	82 (19.6)		33 (2.6)
White sterile mycelium isolate from C.abietis infected needles		188 (21.7)	27 (3.3)	44 (10.7)		43 (3.6)
Cladosporium herbarum	23 (3.7)		123 (18.4)	1 (0.3)	150 (27.3)	622 (53)
Cladosporium cladosporioides (Fresen.) de Vries		17 (3.8)	27 (3.9)		163 (29.6)	40 (3.4)
Aureobasidium pullulans (de Bary) Arnaud	8 (1.2)	26 (5.6)	164 (24.1)	117 (30.9)		

April figures refer to isolates from outer bud scales only.

i) Filamentous fungi

Many different species of filamentous fungi were isolated. The picture presented was complex as the most abundant species in one monthly sample did not necessarily appear in subsequent samples. The most abundant fungal species from each sample are given in Table 45. C. cladosporioides has been included in the September sample from uninfected trees as the abundance of C. herbarum and C. cladosporioides together (22.8%) was greater than that of Humicola sp. Fungi which were isolated from two or more consecutive samples are given in Table 46. This shows that Cladosporium spp. were consistently isolated from both C. abietis infected and uninfected trees. Although Cephalosporium sp. and white sterile mycelium were isolated regularly from trees uninfected by C. abietis, they were only isolated occasionally from the needles taken from uninfected trees. The converse was true for A. pullulans.

The only general seasonal trends which occurred were an overall increase in numbers of Cephalosporium sp. after an initial peak. Most other organisms which appear in Table 45 were isolated only occasionally.

ii) Yeasts

The picture presented by the yeasts is somewhat simpler (Table 47) than that presented by the filamentous fungi. The white yeast Torulopsis sp. was isolated from within the unopened bud, and was the only yeast isolated from newly flushed needles. The pink yeast S. roseus was isolated from both samples of trees from June onwards. This yeast formed the major part of the total yeast population. The white yeast Cryptococcus sp. was isolated from trees infected with C. abietis in July and August. It was more abundant than Torulopsis sp. in the latter sample.

Table 47 Norway spruce: Changing numbers of different yeasts isolated from needles in the period April-September 1971

(Relative numbers g^{-1} fresh weight calculated from proportional sub-sampling data)

Species / Isolate from healthy needles	April Pre Flushing	May Post Flushing	June	July	August	September
Torulopsis sp.	338	959	500			
Sporobolomyces roseus Kluyver & van Niel				260	223	462
Isolate from C. abietis infected needles						
Torulopsis sp.	407	2970	276	40	8	
Cryptococcus sp.				13	30	
Sporobolomyces roseus	273	266	342	545		

April figures refer to isolates from outer bud scales only.
Torulopsis sp. was isolated in very low numbers from within the bud.

Table 48 Norway spruce: Changing numbers of different bacteria isolated from needles in the period April to September 1971

(Relative numbers g^{-1} fresh weight calculated from proportional sub-sampling)

Species isolate from healthy leaves	April Pre flushing	May Post flushing	June	July	August	September
Bacillus sp.	714	466	28	390	140	160
Brevibacterium sp.	20	480	28	262	130	453
Micrococcus sp.					94	173
Achromobacter sp.					47	
Pseudomonas sp.						68
Streptomyces sp.				130		

Species isolate from C.abietis *infected needles*					
Bacillus sp.	610	1400		343	277
Brevibacterium sp.	18	1015		112	817
Micrococcus sp.		1400	86	228	636
Flavobacterium sp.		1518			
Achromobacter sp.		251		285	
Pseudomonas sp.					539
Corynebacterium sp.					94.5
Streptomyces sp.	383	8			

In April Brevibacterium sp. alone isolated from within the bud;

Bacillus sp. isolated from outer bud scales.

iii) Bacteria

In most of the monthly samples the most abundant species of bacteria isolated were Bacillus sp. and Brevibacterium sp. (Table 48). In some samples from C. abietis infected trees, Micrococcus sp. also formed a considerable proportion of the bacterial population. Brevibacterium sp. was the only bacterium isolated from within the unopened bud, whereas Bacillus sp. only was isolated from the outer bud scales. There did not appear to be any marked change in the relative abundance of species throughout the season except the slight increase in the percentage of Brevibacterium sp. isolated.

2) EXPERIMENTS ON C. ABIETIS

a) Spore germination

In all experiments the percentage germination was never greater than 2% under any conditions. The replicate tests were so variable that no reliance could be placed on the very low numbers of positive results obtained. In all tests no germination of teleutospores was ever observed.

b) Infection experiments

All trees used in the infection trials were examined in September and the following March. No symptoms of successful infection by C. abietis were observed in any of the trials.

c) Enumeration of Sporidia in the Airspora

The analysis of airborne particles by use of spore trapping methods is very dependent on the accurate recognition of the particles under study. Before any analysis of spore tapes was attempted sporidia of C. abietis were collected directly from ripe teleutosori using a

needle or small wire loop. The dimensions and appearances of many sporidia were observed to give this final description.

Sporidia of C. abietis are spherical to subglobose, 8-10 µm in diameter but usually 10 µm. The sporidium is orange-yellow in colour and has a very thin spore wall.

Counts of sporidia gained from spore tapes were converted to give numbers per cubic meter of air. The distribution of numbers of sporidia released throughout the sampling period (Fig.54) shows that a definite peak occurred between 21-24 May. An indication of the observation of flushing Norway spruce trees within that compartment is given just above the base line of this figure. The build-up of sporidia release and the flushing time of new needles appears to coincide. The counts of sporidia were done on a four hour basis, which allowed any diurnal fluctuations in sporidia release to be detected. There was a marked regular diurnal periodicity (Fig. 55). This figure uses the combined counts for the equivalent four hour periods of each day during one week. Sporidia release occurred mainly between 2000 hrs. and 0400 hrs. with a peak from 2400 hrs. to 0400 hrs. and rapid fall off by 1200 hrs. This pattern was observed for each week throughout the sampling period except for the final week in which the numbers of sporidia collected were so low that no obvious release pattern could be discerned.

A. PSEUDOPLATANUS/R. ACERINUM

1) ISOLATION AND ENUMERATION OF THE SAPROPHYTIC PHYLLOPLANE MICROFLORA

a) Climatic data

This data was obtained from Langhill Farm Meteorological Station, Midlothian.

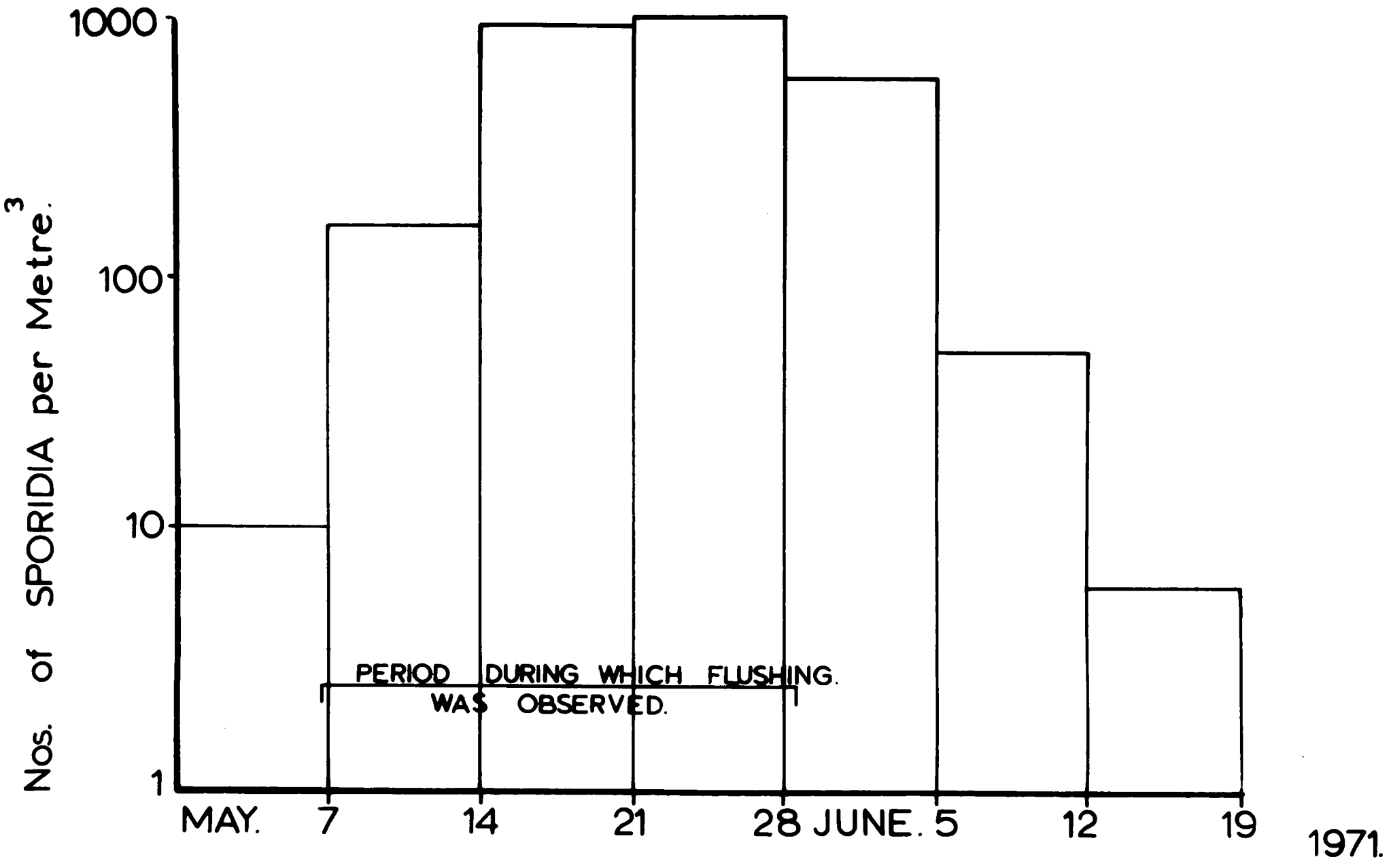


Fig. 54 C. abietis: Numbers of sporidia in air (weekly totals m^{-3})

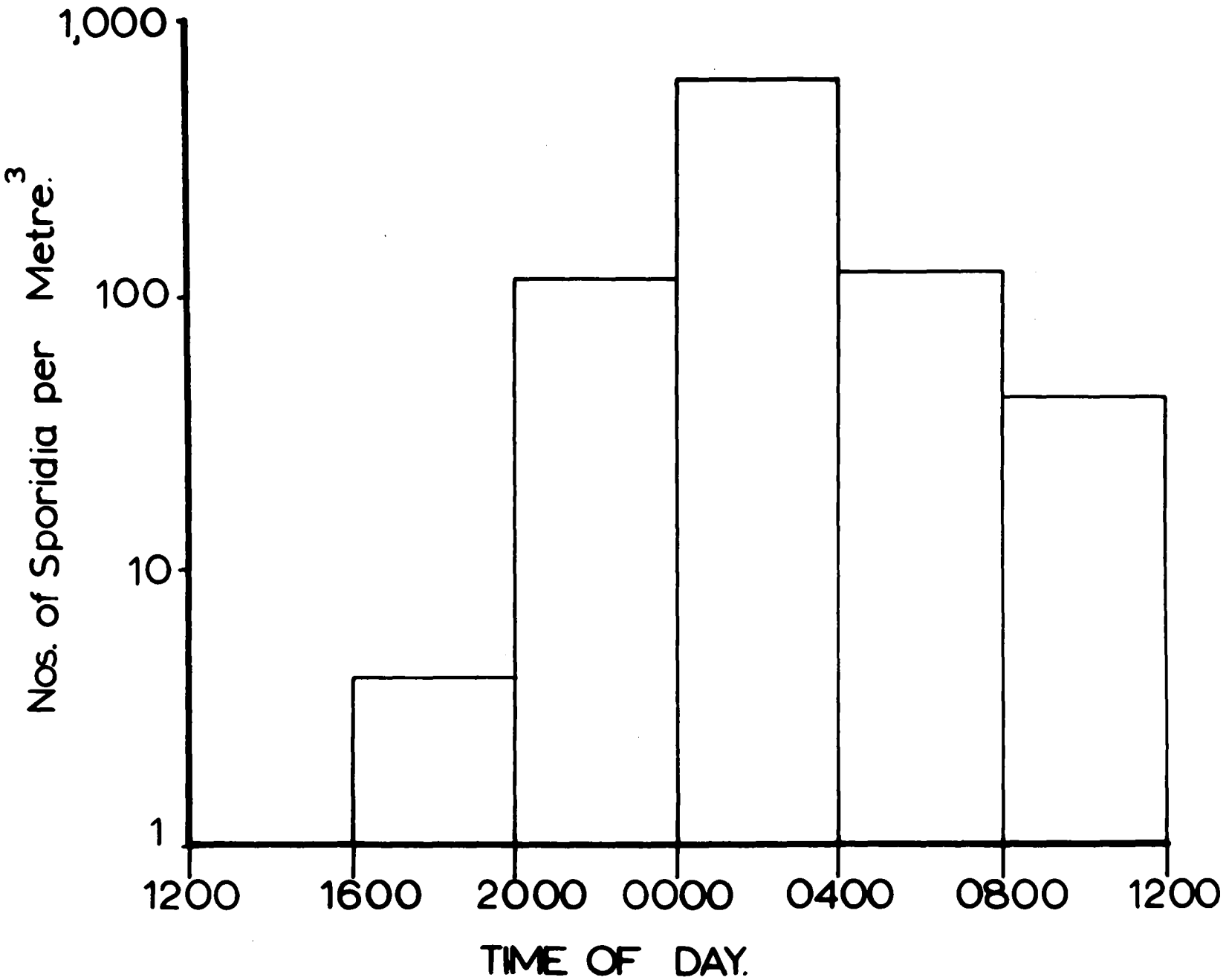


Fig. 55 C. abietis: Diurnal periodicity of sporidia release

i) Temperature

Monthly means of maximum and minimum temperature (Fig. 56) show that a sharp rise in the maximum temperature occurred during May and again during July to rise to a peak of 18.8°C. The minimum temperature rose steadily at a rate of 2.3-2.6°C per month until the end of July.

ii) Rainfall

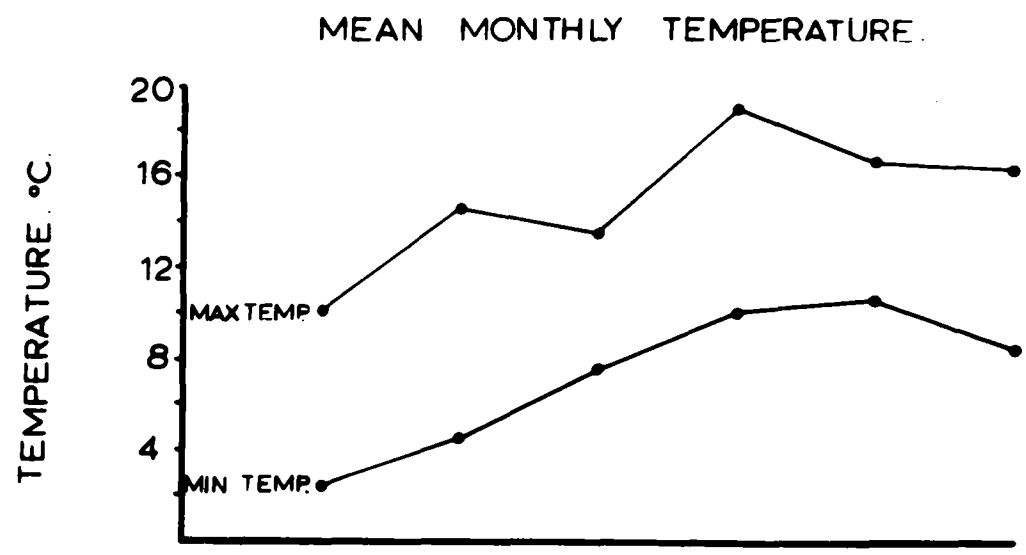
Total rainfall (Fig. 56) shows a similar pattern of increase to the maximum temperature (Fig. 56). The highest total rainfall (112.5 mm) occurred during August and the lowest (25 mm) during September.

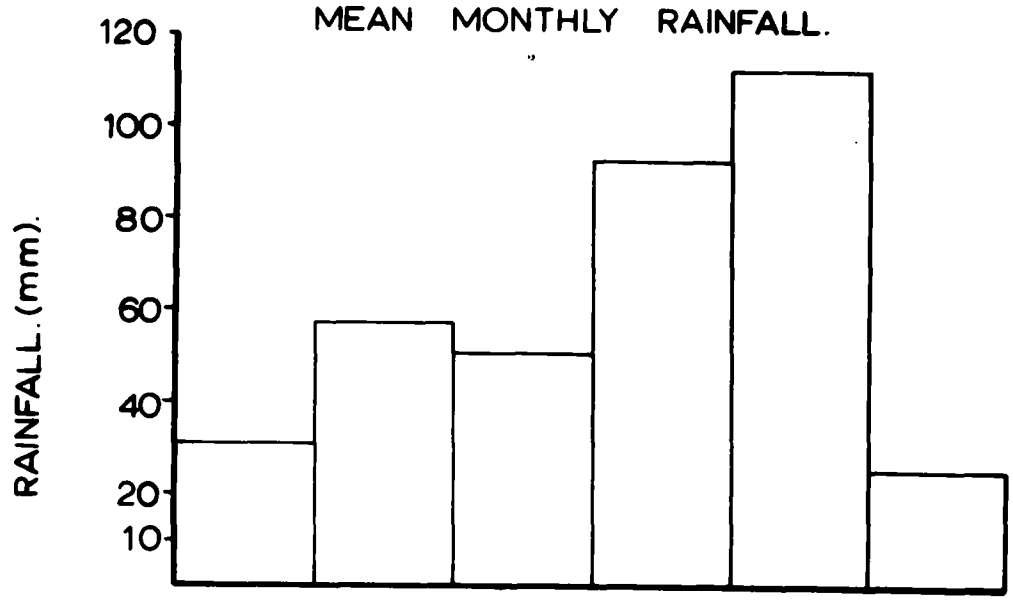
iii) Humidity

Monthly mean relative humidity fluctuates mainly in the range 75-80% R.H. rising to 84% R.H. during September (Fig. 56).

b) Enumeration of the saprophytic microflora of phylloplane

The seasonal pattern observed during the sampling period was an overall increase in numbers of micro-organisms from April to September (Fig. 57). No micro-organisms were isolated from the inner scales of the unopened leaf bud, but low numbers of organisms were isolated from the outer bud scales. Filamentous fungi and yeasts showed a steady increase in numbers throughout the sampling period, whereas numbers of bacteria increased rapidly during June and July, but also fell rapidly during August and maintained their numbers during September. In August and September separate isolations were done on leaves bearing visible symptoms of infection by R. acerinum. Although the numbers of micro-organisms isolated from these leaves were slightly higher than those isolated from uninfected leaves, the only significantly different ($p = .05$) result was for the isolation of yeast cells in the September sample.





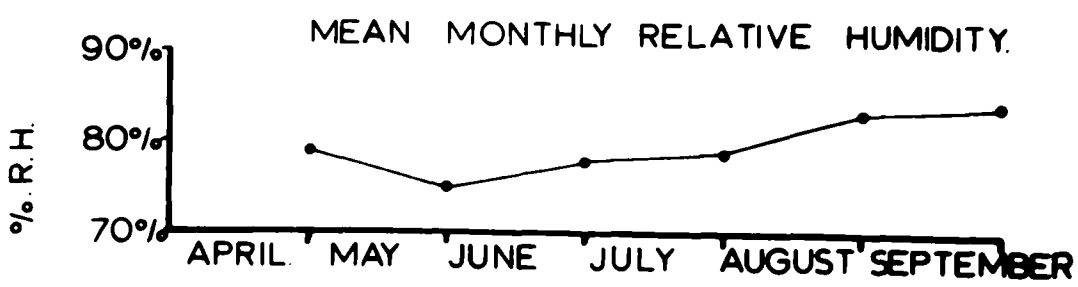


Fig. 56 Meteorological data April to September 1971
Langhill Farm Observatory

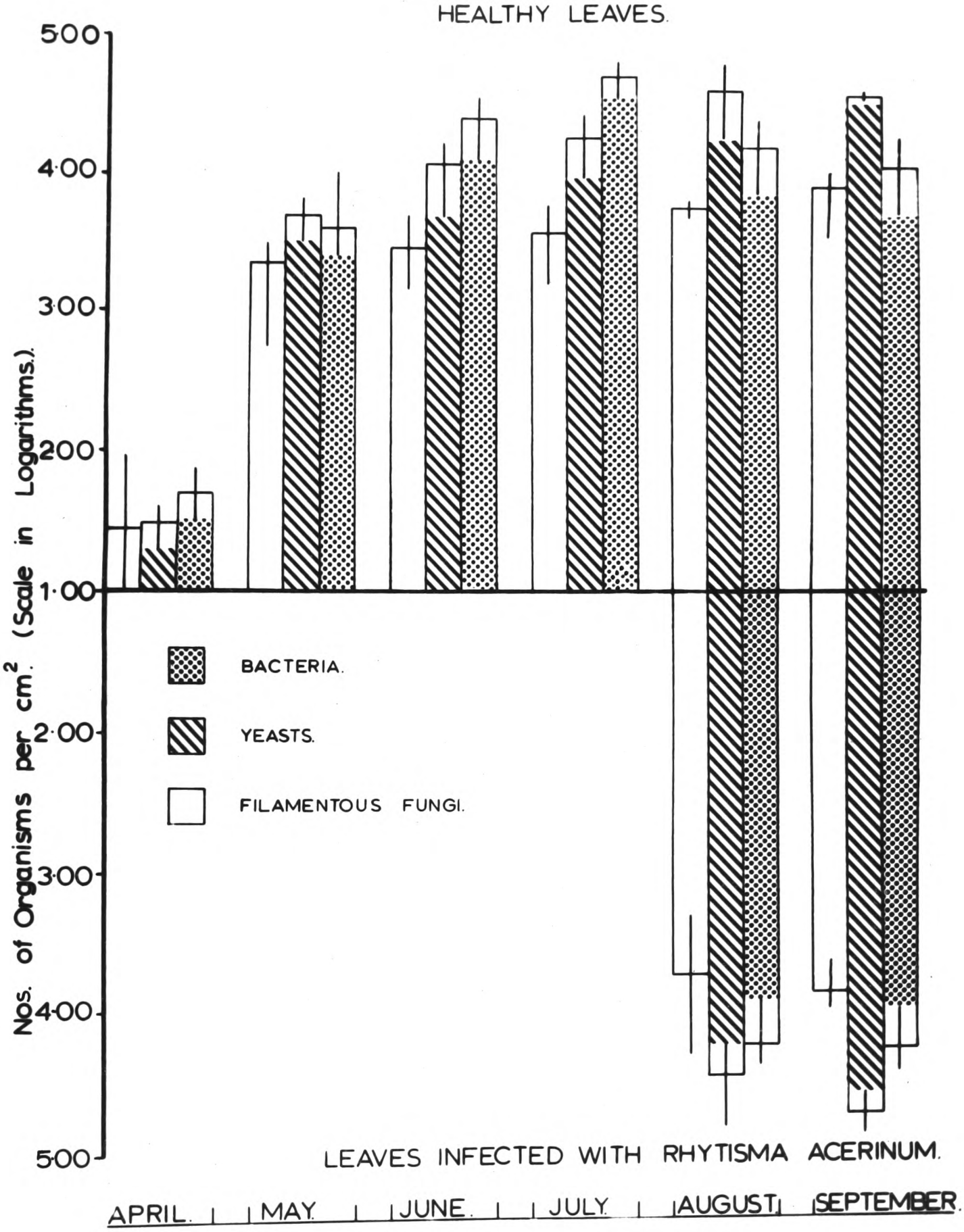


Fig. 57 Sycamore: Numbers of micro-organisms isolated from leaves in 1971

c) Identification of phylloplane micro-organisms

i) Filamentous fungi

A. pullulans was the most abundant fungus present in all samples. This fungus, together with C. herbarum and C. cladosporioides, accounted for 88-100% of the filamentous fungi isolated in all samples. C. cladosporioides was present from May to July whereas C. herbarum increased from low numbers in June to high numbers in September (Table 49). E. nigrum and white sterile mycelia were isolated consistently in low numbers from June or July onwards. In August and September samples from leaves infected by R. acerinum showed a similar distribution of organism species as did the uninfected leaves.

ii) Yeasts

Three species of yeasts were isolated from sycamore leaves during the sampling period (Table 50). Torulopsis sp. was present in the outer bud scales and in all subsequent samples. S. roseus appeared at low numerical levels during May and then increased rapidly during June and July. From July onwards Cryptococcus sp. was isolated in increasing numbers to form a nearly equal proportion of the white yeast population by September. The proportion of the total yeast population formed by the non-pigmented yeasts decreased during June and July but increased again in later samples. In September the numbers of yeast cells isolated from leaves infected by R. acerinum increased significantly ($p = .05$) although the proportion of the total yeast population formed by the non-pigmented yeasts was lower.

iii) Bacteria

Bacillus sp. were isolated from the outer bud scales of sycamore.

Table 49 Sycamore: Changing numbers of different filamentous fungi isolated from leaves in the period April-September 1971

Species	April	May	June	July	August	September
Isolate from healthy leaves						
Alternaria tenuis Nees					63	
Aspergillus fumigatus Fresen.		96				
Aureobasidium pullulans (de Bary) Arnaud	30	1607	1950	1890	2670	2871
Botrytis cinerea Pers. ex Pers.				129		
Cladosporium cladosporioides (Fresen.) de Vries		387	720	660		
Cladosporium herbarum (Pers.) Link ex S.F. Gray			240	1020	2571	3726
Epicoccum nigrum Link ex Schlect.				36	225	240
Fusarium oxysporium Schlect.		160	30			
Mucor genevensis Lend.						30
Penicillium frequentans West.	30					
Phoma sp.					30	123
Sterile white mycelium	30	54			72	189
Isolate from R. acerinum infected leaves						
Alternaria tenuis					54	
Aureobasidium pullulans					2718	2772
Cladosporium herbarum					2610	4098
Epicoccum nigrum					210	213
Mucor genevensis						126
Phoma sp.					30	120
Sterile white mycelium					78	171

April figures are for outer bud scale sample only.

(relative numbers cm^{-2} calculated from proportional sub-sampling data)

Table 50 Sycamore: Changing numbers of different yeasts isolated from leaves in the period April-September 1971

(Relative numbers cm^{-2} calculated from proportional sub-sampling data)

Species	April	May	June	July	August	September
Isolate from healthy leaves						
Torulopsis sp.	30	3550	2100	680	2120	8620
Cryptococcus sp.				300	1130	7760
Sporobolomyces roseus Kluyver & van Niel		1450	9900	17020	19750	20620
Isolate from R. acerinum infected leaves						
Torulopsis sp.					6610	10580
Cryptococcus sp.					1830	8950
Sporobolomyces roseus					21560	38470

April figures refer to isolates from outer bud scales only.

Table 51 Sycamore: Changing numbers of different bacteria isolated from leaves in the period April-September 1971

(relative numbers cm^{-2} calculated from proportional sub-sampling data)

Species	April	May	June	July	August	September
Isolate from healthy leaves						
Bacillus	50	2820	7580	9600	4920	3200
Erwinia sp.	1000	12350	30200	5400	5400	
Achromobacter sp.				5000		
Micrococcus sp.			2000		2000	1000
Flavobacterium sp.					3080	1000
Pseudomonas sp.			2570	5000		400
Isolate from R. acerinum infected leaves						
Bacillus sp.				5390		6460
Erwinia sp.				5770		7990
Micrococcus sp.				2320		1870
Flavobacterium sp.				3130		880
Pseudomonas sp.				1340		2300

April figures refer to isolates from outer bud scales only.

In subsequent samples this Bacillus sp. together with the yellow pigmented rod shaped bacteria formed over 65% of all bacteria isolated (Table 51). The numbers of both of these bacteria increased to a midsummer peak in July, the yellow rod shaped bacteria occurring at about three times the numbers of the Bacillus sp. In August and September the proportional abundance of each bacterial species was nearly the same in isolations from leaves infected or uninfected by R. acerinum.

2) EXPERIMENTS ON R. ACERINUM

The collection of leaves bearing stromata, during the autumn, and the storage of these leaves in cold moist conditions during the winter resulted in ripening of only about 5% of the stromata. The spring collection of stromata on leaves from moist layers of leaf litter gave 30% ripening of the stromata. Ascospores collected from these stromata gave germination percentages never rising above 21% under any condition of light, humidity and temperature. The variability was so great that the only trends that could be inferred were an increase in the germination percentage under temperature conditions of 15-20^{o}C and with a humidity in excess of 98% R.H. No effect due to light was observed. Frequently ascospore suspensions were contaminated by spores of Fusarium spp. The surface sterilisation of the stromata did lower the level of contamination slightly but the stromata treated in this way gave rise to spores showing no germination at all.

In August and September the examination of the infection trials showed that in all cases infection was unsuccessful.

DISCUSSION

Observations on the Norway spruce phylloplane micro-organisms showed that some seasonal trends did occur both in numbers and in species composition. Few micro-organisms were isolated from inner scales of the unopened bud. This has also been observed for buds of many deciduous trees (Warren, personal communication). The micro-organisms on the outer bud scales may have originated from needles remaining on the trees or by deposition from the air. As these bud scales were sticky, the rate of deposition from the air might approach the high levels for this shape of body predicted by aerodynamics (Chamberlain & Chadwick, 1972). During May there was a rapid increase in numbers of microbes isolated from the leaves to give a maximum level after which the numbers declined. This initial peak in microbial numbers has been observed previously for bacteria (Kerling, 1958; Stout, 1960; Leben & Daft, 1967; Hislop & Cox, 1969), and for fungi (Kerling, 1958; Dickinson, 1967; Hislop & Cox, 1969). However, yeast numbers have previously been observed to increase progressively (Dickinson, 1967) which was not apparent in this particular case. It might be thought that this initial peak in microbial numbers was due to a transfer of inoculum from the outer bud scales to the new needles. However, an examination of the species isolated (Appendix 2) shows this to be unlikely, as most of the species which were abundant on bud scales were absent from newly flushed needles, although some carry-over of C. herbarum or A. pullulans may have occurred. Brevibacterium sp., isolated from the inner bud, was responsible for the large increase in bacterial numbers.

Other fluctuations in microbial numbers occurred later in the season. Numbers of filamentous fungi and yeasts did not vary greatly but bacterial numbers dropped sharply in June, and only increased irregularly after that time. These fluctuations in bacterial numbers might have been related to changes in climatic conditions. Many of the samples were taken after dry periods. No rain had fallen in the week prior to the June sample and only 5.5 mm had fallen in the week before that. In this same period the humidity had only reached 90% RH for 22% of the time. The reduction of bacterial numbers on leaf surfaces under dry conditions has been observed on beech leaves (Jensen, 1971) and Leben & Daft (1967) attributed the sparseness of bacteria on plant leaves in Ohio to the short periods for which free water remained on the leaf surface. Preece (1971) stated that when no free water was visible, the relative humidity near the leaf surface approached that of the atmosphere measured by a psychrometer; thus the humidity at the surface of the needles may have been very low. Rain may also affect the bacterial population of the phylloplane by washing organisms from the leaves (diMenna, 1959; Ruinen, 1961), although this effect is thought to be only short term as it may be counteracted by the rapid multiplication of bacteria if the leaf remains wet for 24 hours (Leden & Daft, 1967). Thus it would appear that bacterial numbers could easily have been influenced by the changing climatic conditions, although an overall increase in bacterial numbers was still apparent.

The difference in numbers of saprophytic micro-organisms isolated from trees infected by C. abietis compared to uninfected trees, was only significant ($p = .05$) in the May sample. However, later samples from infected trees did give mean values which were consistently higher in the case of yeasts and bacteria and consistently lower in the case of filamentous fungi. This might indicate that some effect was occurring, but the variation in the isolation technique was such that no quantitative statistical significance could be attached to these small differences. The qualitative analysis of the phylloplane microflora revealed that the most abundant filamentous fungi were A. pullulans which has been isolated from many trees (Table 42) a number of Cladosporium spp. which have been isolated from several trees (Herring, 1965; Hogg & Hudson, 1966; Hislop & Cox, 1969; Pugh & Buckley, 1971a), and Cephalosporium sp. which has previously been isolated most often from soil (Ainsworth & Bisby, 1967). The pink yeast S. roseus comprised the major part of the yeast population from June onwards, at which time it increased rapidly. This phenomenon has already been observed by other workers (Last, 1955a; diMenna, 1959; McBride, 1970; Pugh & Buckley, 1971a). This rapid increase has been attributed to a rise in temperature (diMenna, 1959) and during July a marked increase in temperature from 13.8 to 18.5°C did occur. However, the increase in numbers of organisms has also been correlated to leaf age and the numbers of S. roseus in the airspora at that time (Last, 1955a, b), and so these reasons may be equally applicable in this case. The most abundant filamentous fungi and yeasts isolated from the spruce needles reflect the abundance of these organisms in the airspora (Gregory & Hirst, 1957; Last, 1955b). Bacteria in

the airspora are dominated by gram positive rods, Bacillus spp., and Micrococcus spp. (Fleming, 1908; Zobel, 1942; Pady & Kelly, 1953). Of these only Bacillus sp. was isolated on nearly every occasion. Brevibacterium sp. was isolated from within the bud itself and when the needles flushed its numbers increased to about the same level as Bacillus sp. These yellow pigmented rods isolated in abundance from the needles of Norway spruce have also been isolated in large numbers from beech leaves (Jensen, 1971) where they comprised 30% of the bacteria isolated.

Infection of the needles by Chrysomyxa abietis appeared to exert little effect on the proportions of the different species isolated. A. pullulans and Micrococcus sp. were more common on C. abietis infected needles and the yeast Cryptococcus sp. was only isolated from infected needles. However, the difference in species of micro-organisms which were isolated from C. abietis infected and uninfected trees was of the same order as the differences which Crosse (1965) found between different samples from different apple trees of the same variety. Thus it would appear that during the early stages of infection by C. abietis the infected Norway spruce trees support a phylloplane microflora very similar in quality and quantity to that supported by uninfected trees.

Although C. abietis spore germination experiments were unsuccessful, they serve to illustrate that rust spores may have very precise requirements for the environmental conditions necessary for germination and the formation of viable spores (Sood & Sackston, 1971). The infection trials used young, newly flushed

spruce needles which are supposed to be susceptible to infection by C. abietis at this stage (Delforge, 1908). Peace (1956) observed that successful infection depended on the fine balance between the ripening of fungal spores and the flushing of Norway spruce without considering other environmental conditions, so it was not surprising that these trials were unsuccessful.

Observations on the release of sporidia of C. abietis under field conditions did yield some information. There appeared to be some form of synchronisation between the release of sporidia and the flushing of new needles. This may be regulated by the response of the host plant to springtime changes in temperature and daylength. This should not be taken to mean that sporidia release would be synchronised to the time of needle flushing for all Norway spruce, since one characteristic of this species is considerable variation in the date at which flushing occurs, even within one provenance. Examination of the spore tapes showed that a marked diurnal fluctuation in spore release occurred. This pattern of nightime spore release has been observed for basidiospores of Puccinia malvacearum Mont. (Carter & Banyer, 1964) and sporidia of Cronartium ribicola Fischer (van Arsdel, 1967). The latter author also observed that spores released at night encountered different environmental conditions to spores released during the day. Many spores released at night are smaller and lighter. Such spores disperse more readily on the low speed stable nocturnal winds (Hirst, 1953). The sporidia of C. ribicola are of similar dimensions and wall thickness as those of C. abietis. It might be reasonable to infer that since the sporidia of C. ribicola are light sensitive and somewhat susceptible to drying (van Arsdel,1967)

that the sporidia of C. abietis might be sensitive in a similar manner. This possible sensitivity may explain to some extent the failure of sporidia to germinate or infect Norway spruce trees in the infection trials.

The results of the isolation of micro-organisms from the phylloplane of sycamore presented a simpler picture than that described for Norway spruce. No micro-organisms were found in the inner bud but filamentous fungi, yeasts and bacteria were isolated from the outer bud scales. This is in agreement with the earlier work of Pugh and Buckley (1971b) who isolated A. pullulans from the outer bud scales of this plant. From May onwards a steady increase in numbers of yeasts and filamentous fungi occurred throughout the sampling period, again in general agreement with the earlier work of Pugh and Buckley (1971a) on sycamore leaves, although the figures obtained here were not directly comparable as they were not expressed in the same manner. The numbers of bacteria increased to a peak in July and then declined. It was possible that the high rainfall in this month (Fig. 56) had caused a temporary decline in bacterial numbers, also observed by other workers (diMenna, 1959; McBride, 1970), but this would not explain the further decline in numbers that occurred in September when the rainfall was lower. However, the humidity was high in this month (Fig. 56) and so it would be unlikely that conditions would be so dry as to lead to a reduction of bacterial numbers similar to that observed on beech leaves by Jensen (1971).

The numbers of species of filamentous fungi isolated from sycamore was smaller than the number isolated from Norway spruce. This may reflect the difference that the shape of leaf exerts on the ability of the components of the airspora to be deposited onto the leaf surface (Gregory, 1961), as many of the species isolated from Norway spruce were abundant in the airspora (Gregory & Hirst, 1957). The major fungal components of the phylloplane microflora isolated from these sycamore trees in Midlothian were similar to those isolated from sycamores in Nottinghamshire (Buckley, 1971). Thus, this component of the microflora appeared to show little variation due to site. The seasonal variation in white and pink yeast numbers was similar to that described by Hislop and Cox (1969), in that the white yeasts decline in relative abundance during the mid-summer months and increase in the autumn. This pattern was partly due to a decrease in the numbers of white yeasts but the major change was due to the rapid increase in numbers of pink yeasts during July. Such increases in these yeasts have been observed previously (Last, 1955a; Hislop & Cox, 1969; McBride, 1970; Pugh & Buckley, 1971a). During August and September Cryptococcus sp. was isolated and increased in numbers to form about 50% of the white yeast population. McBride (1970) observed similar changes in the relative proportions of Cryptococcus sp. and Torulopsis sp.

In September the number of yeasts isolated from leaves infected by Rhytisma acerinum increased significantly ($p = .05$), relative to the uninfected leaves, owing to the increase in numbers of S. roseus isolated (20×10^3 to $38 \times 10^3 cm^{-2}$). A similar pattern of increase in numbers of S. roseus, from 0.5 to 21 cm^{-2}, has been observed,

using the sporefall technique, on apple leaves infected by Podosphaera leucotricha (Ell. & Everh) Salm. (Last,1970). The most abundant species of bacterium isolated was Erminia herbicola. The preponderance of these yellow pigmented bacteria on the phylloplane has been observed by many authors (Last & Deighton, 1965; Leben, 1965a; Jensen, 1971).

The failure of ascospores of R. acerinum to germinate might be attributable to the sensitivity of the stromata and ascospores to dry conditions (Jones, 1925). When many of the ascospore suspensions were found to be contaminated by Fusarium spp., it was suspected that these contaminating fungi may well have exhibited some antagonism to R. acerinum. Several Fusarium spp. have been shown to be antagonistic to other fungi (Davis, 1968; Wensley, 1969). Surface sterilisation did reduce the incidence of contamination, but asospores of R. acerinum produced by these stromata were non viable. This sensitivity to chemicals may well indicate a possible reason for the failure of the infection trials. These infection experiments were done using trees at King's Buildings, Edinburgh. It may well be that the atmosphere of the city is polluted to a sufficient level to prevent ascospore germination and infection of the sycamore leaves. Peace (1962) observed that R. acerinum was very susceptible to atmospheric pollution. This aside, other environmental conditions which may be necessary for successful infection are not known although Massée (1901) managed to achieve successful infection of several Acer spp. by spraying the leaves with a spore suspension.

The variable low level of germination of either R. acerinum or C. abietis, together with the failure to achieve successful infection of the host plants under experimental conditions led to the view that these combinations of host/pathogen system were not suitable for an initial study of the interaction of phylloplane saprophytes with pathogens and with the host plants. These results are presented here as a record of the surveys of the saprophytic micro-organism populations on the phylloplane of Norway spruce and sycamore and the effect that the infection of these leaves exert on these populations.

GENERAL DISCUSSION

GENERAL DISCUSSION

The patterns of increase in numbers of saprophytic phylloplane micro-organisms have been observed on field plants by many other workers. Such studies have been extensively reviewed (Last & Deighton, 1965; Hudson, 1968; Last & Warren, 1972). The gradual increase in microbial numbers on sycamore leaves observed by Pugh & Buckley (1971a) was confirmed in the present study. The pattern in which low numbers of micro-organisms were isolated from leaves until July, previously observed on several plants (Last & Deighton, 1965) was also observed in the isolations from antirrhinums. However, the pattern of a large initial increase in numbers of phylloplane micro-organisms immediately after bud break on the needles of Norway spruce was somewhat different although a similar burst of activity has been observed on Lime (Tilia europea L.) (Warren personal communication). In no case was there evidence for a successional colonisation of the leaf surface by major groupings of micro-organisms similar to that observed by Ruinen (1961) and McBride (1970).

The effect of infection by a pathogen on the saprophytic phylloplane microflora of the leaf was similar on sycamore and antirrhinum leaves infected by Rhytisma acerinum and Puccinia antirrhini respectively. A large increase in numbers of organisms, especially yeasts, occurred at the time the pathogen ruptured the leaf epidermis. This has been observed on other plant leaves (Last, 1970). The observations on Norway spruce are not comparable, as no isolations were made at the time when sori of Chrysomyxa abietis ruptured the epidermis of the needles in the spring after overwintering within the needles.

Neither of the tree pathogens proved to be amenable to use in laboratory experiments and so more detailed studies were done using the Antirrhinum majus / Puccinia antirrhini system. The most abundant saprophytes isolated from antirrhinum leaves were Sporobolomyces roseus and Cladosporium cladosporioides which have been found in high numbers on many other plant species (Last & Deighton, 1965; Last & Warren, 1972). As the ability of saprophytes to persist in high numbers on leaves has been found to be important in interactions on the phylloplane (Goodman, 1967), these organisms were used in studies on leaf colonisation and pathogen saprophyte interaction.

The ability of these organisms to colonise green leaves was observed in experiments using a model system comprising single rooted detached leaves growing in petri dishes. Using this cultural method and controlling the environment within fine limits enabled observations to be made on some of the factors affecting colonisation patterns. This technique made it possible to distinguish the effect of leaf age alone from the combined effects of season and leaf age observed on field plants both by other workers (Kerling, 1958; Ruinen, 1961) and also in this study (p37). Early work by Last (1955a) related the increase in numbers of S. roseus to the age of leaves and these observations were confirmed in this study, as the total volume of cells of S. roseus was significantly greater ($p = .05$) on older leaves of either cultivar of antirrhinum. Not only leaf age but also cultivar of antirrhinum was found to affect the colonisation of older leaves (Fig. 30), greater volumes of cells being attained on A. Nanum leaves.

The colonisation of leaves by C. cladosporioides was also found to be affected by leaf age and cultivar of antirrhinum, in that the production of secondary spores was only observed on older leaves. The

associated reduction in the overall percentage spore germination was observed to occur two days earlier on A. Nanum leaves as compared to A. Fi hybrid leaves. Towards the end of the experiments (21 days) the length of germ tubes on equivalent areas of the leaf was greater on A. Nanum leaves.

Such differences were not observed on field plants possibly due to the differences in planting dates and the variability of results between groups of isolations using these plants. These differences in the colonisation of the two cultivars correlate well with the difference in predicted nutritional status of the leaves in terms of carbohydrates, as determined by the analysis of leaf leachates (Table 17), and the effects of exogenous nutrients on these saprophytes (Fig. 16; Table 14). The quantities of carbohydrates leached from the younger leaves of either cultivar of antirrhinum were similar and also lower than the amount leached from older leaves. The leaf washings from the older leaves of A. Nanum contained about ten times the quantity of carbohydrates leached from A. Fi hybrid leaves of the same physiologic age.

A second factor which was observed using this experimental system was the differential effect of different areas of the leaf on C. cladosporioides. The veinal distribution of fungi has been observed on sycamore leaves in the field (Pugh & Buckley, 1971a), and in these experiments the midrib of older antirrhinum leaves was found to stimulate the development of C. cladosporioides relative to the leaf lamina. However, even under these controlled conditions, it was not possible to place statistical significance on the appreciable differences in germination although later tests did show such

significance for germ tube growth. The washing technique used to reisolate S. roseus did not allow observations to be made in order to see if the yeast was affected in a similar manner. However, concentrations of yeast cells were observed above the anticlinal walls of epidermal cells of the leaf lamina which have been observed previously on leaves of other plants (Last, 1955a; McBride, 1970).

A consistent mutual antagonism was observed between S. roseus and C. cladosporioides both in experiments on leaf colonisation and in experiments with P. antirrhini. The interactions between all three organisms on rooted detached leaves presented a very complex picture. On leaves the interactions were not the same as those predicted by the in vitro tests in which S. roseus inhibited P. antirrhini and C. cladosporioides stimulated the rust. In detached leaf culture S. roseus tended to stimulate P. antirrhini whereas C. cladosporioides inhibited the rust. Such differences between in vitro and in vivo tests have been observed by other workers using different combinations of pathogen and host plant (Akai & Kuromota, 1968; van den Heuvel, 1969) and these have been attributed to the formation of phytoalexins on the leaf surface triggered by the antagonising organisms (Bailey, 1971). This hypothesis referred to experiments in which the pathogen was inoculated onto the leaf some time after the saprophytes which might be expected to predispose or immunise the leaves to some extent (Matta, 1971). However in these experiments simultaneous inoculation was used. Phytoalexins have been observed to inhibit saprophytes more severely than pathogens (Cruickshank, 1962) and so, if C. cladosporioides had induced phytoalexin production, it might be expected that this

micro-organism would not develop well on the leaf surface. However, spore germination and sporulation of C. cladosporioides have been observed on the surface of antirrhinum leaves and so phytoalexins may not be implicated in the interaction with P. antirrhini, or may not play a major role in the inhibitions observed when these two organisms were incubated together on the leaf surface. Nevertheless the leaf surface should not be regarded as inert, as in vitro experiments using leaf leachates from uninfected plants revealed that saprophytic micro-organisms were stimulated by these solutions whereas P. antirrhini was inhibited by leachates especially those from A. Fi hybrid (Table 21). This inhibitor appeared to be preformed in that it did not require the presence of a micro-organism to trigger its formation and may therefore be distinct from the phytoalexin mechanisms discussed above.

A review of work on preformed substances on leaves which were antagonistic to pathogens was given by Wood (1967) who raised the point that there could be a considerable difference in pathogen response to extracted substances in vitro and the conditions achieved in vivo. Although the development of P. antirrhini on the leaf was better than that predicted by the in vitro tests with leaf leachates, the development on the leaves of the resistant A. Fi hybrid was significantly less than that on the susceptible A. Nanum under optimal conditions for rust spore development. The difference between the in vitro and in vivo tests might be due to a thigmotrophic stimulation of P. antirrhini by the leaf surface which has been observed for spores of other rust species (Dickinson, 1949), or the difference between the concentration of substances on the leaf surface and the

calculated concentration used to obtain the leaf leachate solutions (Kovacs & Szeöke,1956).

Although the combination of rooted detached leaf culture and the P. antirrhini / A. majus system successfully enabled detailed observations to be made on the initial stages of leaf colonisation by two commonly isolated phylloplane saprophytes and the interactions between these micro-organisms and an obligate pathogen, the results obtained in the latter case need further elucidation as to the nature of the interactions between saprophytes, pathogens and the host plant as no clear pattern was observed in these experiments. This will involve not only the culturing of rooted detached leaves, but also whole plants grown under aseptic and controlled environmental conditions. The effects of the conditioning of leaves by saprophytes prior to pathogen attack require study as does the effect of saprophytes on the leaf surface on any inhibitory compounds discovered during the further analysis of leaf leachates. Neither the nature of the observed interactions between P. antirrhini, C. cladosporioides and S. roseus, nor the effect of environmental conditions on such interactions should be overlooked in future studies to further refine the model system in order to attempt to answer some of the questions raised by this study.

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APPENDICES

APPENDIX 1

Data on leaf colonisation experiments

and microbial interactions in Section 3

S. roseus: Volume of cells per leaf of A. Nanum and A. Fi hybrid isolated from attached leaves: Experiment 1

CULTIVAR	TREATMENT	MEASURE	Volume of cells ($\mu m^3 \times 10^3$) per leaf INCUBATION PERIOD (DAYS) 0	1	2	4	7	14	21
A. Nanum	S. roseus alone	Mean	251.2	4363.2	3969.6	4132.8	11472	17688	16632
		Min.	1351.2	0	0	40.8	0	0	35.5
		Max.	3295.2	10888.8	18432	26222.4	50592	56016	36168
	S. roseus + C. cladosporioides	Mean	341.5	2786.4	2011	3288	5080.2	10392	9849.6
		Min.	126.4	0	68.8	30.2	0	117.3	0
		Max.	461.5	9576	4857.4	26428.8	25411.2	53520	39672
A. Fi hybrid	S. roseus alone	Mean	344.16	2379.4	933.1	7458.9	6588	8294.4	18513.6
		Min.	129.6	0	30	0	492	0	0
		Max.	844.8	11740.8	7250.4	38503.2	35088	29932.8	83076.0
	S. roseus + C. cladosporioides	Mean	378.72	1632	2556	3691.2	4135.2	3612	13881.6
		Min.	154	0	164.8	33.12	1759.2	199.2	0
		Max.	962.4	3573.4	11032	13281.6	8464.8	6621.6	67348.8

S. roseus: Volume of cells per leaf of A. Nanum and A. Fi hybrid isolated from attached leaves: Experiment 2

CULTIVAR	TREATMENT	MEASURE	Volume of cells ($\mu m^3 \times 10^3$) per leaf INCUBATION PERIOD (DAYS) 0	1	2	4	7	14	21
A. Nanum	S. roseus alone	Mean	197.8	2983.2	2635.2	5532	1036.8	10476	20323
		Min.	129.6	201.6	316.8	0	0	58.32	0
		Max.	247.2	12770	45516	35424	3600	52212	117072
	S. roseus + C. cladosporioides	Mean	183.4	1039.2	3333.6	4903.2	10699	7308	9292.8
		Min.	152.9	352.8	98.4	0	0	0	0
		Max.	288.96	4300.8	126000	27360	65520	35760	52416
A. Fi hybrid	S. roseus alone	Mean	414	3192	3048	7056	6168	24576	14088
		Min.	175.2	297.6	95.6	24	0	213.6	0
		Max.	492	1344	91200	38880	21024	127200	80640
	S. roseus + C. cladosporioides	Mean	381.6	1051.2	1500	4418	1420.8	3528	7776
		Min.	213.6	511.2	171.6	331.2	0	0	0
		Max.	8328	6936	11808	17280	15336	22728	45360

C. cladosporioides: Percentage spore germination on leaves of A. Nanum

Experiment 1

POSITION ON LEAF	TREATMENT	MEASURE	INCUBATION PERIOD (DAYS) 0	1	2	4	7	14	21
Lamina	C. cladosporioides	Mean	0	48.2	27.5	43.2	52.0	50.1	40.2
		Min.		0	0	0	35.0	14.6	0
		Max.		82.0	74.8	68.0	67.5	70.1	60.2
	C. cladosporioides + S. roseus	Mean	0	42.0	49.8	37.5	44.4	44.2	36.0
		Min.		30.0	9.5	0	0	13.8	0
		Max.		54.3	78.4	45.7	72.0	80.0	60.4
Midrib	C. cladosporioides alone	Mean	0	50.1	25.5	56.3	52.0	40.4	34.5
		Min.		36.0	0	44.3	0	0	0
		Max.		87.2	52.4	70.0	85.3	64.6	60.0
	C. cladosporioides + S. roseus	Mean	0	45.8	61.0	45.0	56.3	35.2	42.1
		Min.		31.8	38.0	22.4	45.1	0	0
		Max.		59.5	74.3	70.8	70.0	64.3	70.1

C. cladosporioides: Percentage spore germination on leaves of A. Nanum
Experiment 2

POSITION ON LEAF	TREATMENT	MEASURE	INCUBATION PERIOD (DAYS) 0	1	2	4	7	14	21
Lamina	C. cladosporioides alone	Mean	0	46.4	45.3	48.2	45.2	47.1	40.0
		Min.		0	0	17.4	0	0	0
		Max.		89.3	73.4	69.5	65.3	72.1	69.8
	C. cladosporioides + S. roseus	Mean	0	40.1	43.4	45.3	46.2	44.0	33.2
		Min.		0	23.1	0	0	0	17.4
		Max.		79.8	63.2	89.4	64.3	77.0	69.8
Midrib	C. cladosporioides alone	Mean	0	54.6	58.6	58.2	48.4	40.3	37.6
		Min.		42	0	38.4	0	32.4	0
		Max.		84.7	89.3	77.8	63.2	61.4	68.2
	C. cladosporioides + S. roseus	Mean	0	47.8	59.3	57.8	49.7	43.2	39.8
		Min.		21.4	0	16.1	0	0	15.3
		Max.		77.8	82.9	74.3	61.4	63.8	59.7

C. cladosporioides: Percentage spore germination on leaves of A. Fi hybrid
Experiment 1

POSITION ON LEAF	TREATMENT	MEASURE	INCUBATION PERIOD (DAYS) 0	1	2	4	7	14	21
Lamina	C. cladosporioides alone	Mean	0	20.0	44.8	46.5	35.2	36.0	32.0
		Min.		0	15.0	0	0	0	10.2
		Max.		45.8	62.0	71.8	62.0	73.6	59.4
	C. cladosporioides + S. roseus	Mean	0	18.3	24.8	31.9	34.0	36.0	37.8
		Min.		0	12.1	0	0	0	0
		Max.		59.0	33.7	70.0	77.9	72.0	60.5
Midrib	C. cladosporioides alone	Mean	0	38.0	50.0	54.3	44.3	18.7	18.4
		Min.		14.8	0	0	22.9	0	0
		Max.		54.4	77.6	81.0	74.0	75.3	49.7
	C. cladosporioides + S. roseus	Mean	0	35.8	31.1	52.4	30.0	37.6	30.2
		Min.		12.0	0	37.3	0	0	0
		Max.		58.2	40.0	80.7	54.9	69.8	50.1

C. cladosporioides: Percentage spore germination on leaves of A.Fi hybrid
Experiment 2

POSITION ON LEAF	TREATMENT	MEASURE	INCUBATION PERIOD (DAYS) 0	1	2	4	7	14	21
Lamina	C. cladosporioides alone	Mean	0	23.4	41.2	44.7	38.3	31.4	32.5
		Min.		0	0	0	12.4	0	7.2
		Max.		53.7	62.4	73.8	61.5	47.6	59.0
	C. cladosporioides + S. roseus	Mean	0	16.3	25.7	34.3	39.8	38.7	39.4
		Min.		0	14.3	0	12.1	0	0
		Max.		49.2	53.4	46.0	52.7	63.9	71.4
Midrib	C. cladosporioides alone	Mean	0	41.3	47.4	49.5	42.3	28.7	17.2
		Min.		0	0	14.1	8.6	0	0
		Max.		68.4	73.4	69.2	64.3	48.7	47.3
	C. cladosporioides + S. roseus	Mean	0	37.6	32.3	44.9	57.2	32.4	33.6
		Min.		0	0	0	18.3	0	5.1
		Max.		58.2	47.3	87.4	83.1	62.7	59.1

C. cladosporioides: Germ tube growth on leaves of A. Nanum

Experiment 1

POSITION ON LEAF	TREATMENT	MEASURE	Germ tube length (μm) INCUBATION PERIOD (DAYS) 0	1	2	4	7	14	21
Lamina	C. cladosporioides alone	Mean	0	16.9	9.81	14.8	20.2	58.2	177.4
		Min.		3.3	7.9	7.9	15.6	37.4	122.4
		Max.		25.7	11.3	26.5	22.2	65.7	259.3
	C. cladosporioides + S. roseus	Mean	0	12.1	13. 4	11.5	19.25	38.5	115.1
		Min.		8.4	5.6	9.5	10.2	32.5	58.7
		Max.		15.1	19.4	17.5	29.5	43.7	143.2
Midrib	C. cladosporioides alone	Mean	0	17.2	9.5	16.5	25.9	68.0	194.9
		Min.		9.9	9.4	8.8	24.8	40.3	176.2
		Max.		24.9	9.7	31.4	26.9	79.4	243.5
	C. cladosporioides + S. roseus	Mean	0	13.8	17.7	12.2	22.6	44.5	166.6
		Min.		9.8	8.7	10.3	10.1	36.9	105.3
		Max.		16.0	23.4	14.2	36.7	52.1	189.4

C. cladosporioides: Germ tube growth on leaves of A. Nanum
Experiment 2

POSITION ON LEAF	TREATMENT	MEASURE	Germ tube length (µm) INCUBATION PERIOD (DAYS) 0	1	2	4	7	14	21
Lamina	C. cladosporioides alone	Mean	0	11.4	12.8	12.1	24.7	42.4	158.4
		Min.		7.2	8.5	10.6	16.7	34.3	104.7
		Max.		14.9	16.2	18.5	27.3	49.8	176.3
	C. cladosporioides + S. roseus	Mean	0	8.7	9.8	11.5	20.1	34.8	105.4
		Min.		6.4	6.5	9.6	16.4	28.8	56.2
		Max.		13.8	14.3	18.1	24.9	41.2	149.3
Midrib	C. cladesporioides alone	Mean	0	13.5	14.9	14.0	28.2	43.9	173.2
		Min.		8.6	12.2	11.5	13.8	35.5	126.4
		Max.		16.3	17.3	18.9	37.4	50.1	193.2
	C. cladesporioides + S. roseus	Mean	0	11.9	12.6	13.6	24.8	53.1	123.5
		Min.		8.4	8.6	4.1	21.8	34.2	89.2
		Max.		14.1	16.1	22.4	29.2	68.9	184.1

C. cladosporioides: Germ tube growth on leaves of A. Fi hybrid
Experiment 1

POSITION ON LEAF	TREATMENT	MEASURE	Germ tube length (μ m) INCUBATION PERIOD (DAYS) 0	1	2	4	7	14	21
Lamina	C. cladosporioides alone	Mean	0	9.9	18.0	12.1	26.0	29.9	120.5
		Min.		7.9	10.4	10.3	9.4	12.7	89.3
		Max.		13.5	30.3	15.2	35.6	47.2	141.7
	C. cladosporioides + S. roseus	Mean	0	7.1	11.1	15.3	21.2	25.6	84.3
		Min.		5.1	6.8	10.2	11.3	22.3	62.4
		Max.		8.2	14.6	23.0	27.0	29.8	100.5
Midrib	C. cladosporioides alone	Mean	0	10.1	19.2	15.6	30.8	32.8	158.4
		Min.		7.3	11	11.6	12.2	12.7	122.5
		Max.		13.5	36.8	21.5	42.9	52.9	197.0
	C. cladosporioides + S. roseus	Mean	0	18.9	10.5	5.6	27.0	33.5	110.9
		Min.		4.2	8.7	10.6	11.6	23.9	84.6
		Max.		43	13.4	22.1	50.0	49.1	157.5

C. cladosporioides: Germ tube growth on leaves of A. Fi hybrid
Experiment 2

POSITION ON LEAF	TREATMENT	MEASURE	Germ tube length (µm) INCUBATION PERIOD (DAYS) 0	1	2	4	7	14	21
Lamina	C. cladosporioides alone	Mean	0	9.2	16.2	18.7	25.4	43.2	101.4
		Min.		7.0	14.7	11.5	16.7	38.9	87.6
		Max.		14.7	19.6	28.2	34.6	53.2	193.2
	C. cladosporioides + S. roseus	Mean	0	6.7	15.6	19.5	24.7	38.9	75.5
		Min.		3.3	12.5	14.8	11.3	29.3	50.1
		Max.		15.7	18.9	23.7	29.4	42.4	89.4
Midrib	C. cladosporioides alone	Mean	0	10.9	12.6	16.7	24.7	59.4	142.3
		Min.		6.4	8.5	10.7	14.2	37.6	182.4
		Max.		15.9	19.7	24.8	37.5	72.1	193.2
	C. cladosporioides + S. roseus	Mean	0	8.8	10.8	17.8	20.3	43.8	84.7
		Min.		3.5	7.4	12.4	15.8	34.6	68.9
		Max.		12.3	19.3	28.2	28.2	51.7	122.4

S. roseus: Total volume of cells (μm^3) cm^{-2} x 10^5 produced on detached older leaves (LPI 1.5) of A. Nanum

TREATMENT	MEASURE	INCUBATION PERIOD (DAYS) 0	1	2	4	5	6	7	14	21
S. roseus alone	Mean	37.3	5.1	10.23	23.01	74.44	135.50	189.35	462.20	692.70
Exp. 1	S.D.	0.35	0.95	4.03	10.96	56.4	116.3	46.7	176.1	142.2
Exp. 2	Mean	2.36	4.69	20.40	12.45	49.47	120.93	207.60	473.47	718.03
	S.D.	0.4	1.3	9.4	5.9	1.3	29.4	106.0	56.7	269.2
Exp. 3	Mean	4.47	8.79	12.32	46.50	97.29	208.10	356.62	703.45	992.57
	S.D.	0.7	3.5	7.2	19.5	18.2	41.4	57.1	200.6	959.0
S. roseus + C. cladosporioides	Mean	3.35	3.46	3.29	3.39	3.20	3.56	3.93	51.83	151.43
Exp. 1	S.D.	0.8	0.9	0.9	0.8	1.4	1.1	0.8	11.7	88.3
Exp. 2	Mean	1.82	2.35	2.57	3.06	2.92	3.65	4.29	84.90	223.00
	S.D.	0.7	1.2	1.4	0.7	1.2	0.9	1.2	30.0	155.6
Exp. 3	Mean	4.70	7.76	6.42	7.58	8.18	8.43	9.49	156.55	214.51
	S.D.	0.8	6.6	1.5	2.0	5.3	5.0	4.0	288.5	148.0

S. roseus: Total volume of cells (μm^3) cm^{-2} x 10^5 produced on detached older leaves (LPI 1.5) of A. Fihybrid

TREATMENT	MEASURE	INCUBATION PERIOD (DAYS) 0	1	2	4	5	6	7	14	21
S. roseus alone	Mean	3.20	6.15	9.78	19.56	54.78	98.72	190.32	288.35	432.81
Exp. 1	S.D.	0.4	0.6	2.1	4.3	8.1	27.9	66.9	30.0	8.6
Exp. 2	Mean	2.56	6.64	12.57	32.59	89.81	175.20	214.72	333.47	421.71
	S.D.	0.4	0.4	0.7	9.5	39.7	93.4	60.1	85.3	104.2
Exp. 3	Mean	1.45	3.42	5.16	15.17	35.67	125.44	182.52	215.77	288.54
	S.D.	0.2	0.6	0.4	1.6	6.8	49.9	87.4	108.5	134.6
S. roseus + C. cladosporioides	Mean	3.39	3.57	3.52	2.94	3.00	1.62	3.79	81.60	108.11
Exp. 1	S.D.	0.3	0.4	1.3	0.7	1.0	0.7	1.0	55.7	86.6
Exp. 2	Mean	2.33	3.03	3.00	2.97	2.73	3.16	3.58	78.36	168.69
	S.D.	0.6	0.7	0.8	0.7	0.9	0.2	0.9	40.5	130.6
Exp. 3	Mean	1.38	1.88	2.62	1.84	2.17	2.39	2.61	40.8	67.21
	S.D.	0.3	0.6	0.5	0.4	0.5	0.6	0.8	14.6	21.0

S. roseus: Total volume of cells (μm^3) cm^{-2} x 10^5 produced on detached young leaves (LPI -0.5) of A. Nanum

TREATMENT	MEASURE	INCUBATION PERIOD (DAYS) 0	1	2	4	5	6	7	14	21
S. roseus alone	Mean	3.31	4.43	8.26	11.55	37.86	65.10	98.55	130.00	115.00
Exp. 1	S.D.	1.17	1.62	2.15	4.15	20.35	43.66	42.35	62.71	54.99
Exp. 2	Mean	1.63	4.69	7.82	8.35	21.45	22.94	36.83	64.25	31.73
	S.D.	0.8	2.33	4.83	5.61	10.89	5.12	26.76	31.84	59.4
Exp. 3	Mean	2.35	4.63	6.52	9.27	34.41	52.56	58.54	76.88	99.84
	S.D.	1.79	2.31	3.63	3.20	16.35	30.27	25.63	49.87	41.32
S. roseus + C. cladosporioides	Mean	3.19	3.61	5.20	7.00	6.70	6.66	18.33	39.07	31.84
Exp. 1	S.D.	2.32	3.12	2.83	4.53	3.25	1.95	14.85	44.75	32.8
Exp. 2	Mean	1.56	1.48	3.41	3.52	3.08	3.16	11.57	23.39	17.26
	S.D.	1.24	0.95	1.65	2.98	2.39	0.67	10.19	13.08	19.6
Exp. 3	Mean	2.32	3.75	2.29	4.47	4.26	4.84	7.56	31.22	16.77
	S.D.	1.05	1.8	1.54	2.56	3.13	2.61	3.37	24.66	25.48

S. roseus: Total volume of cells (μm^3) cm^{-2} x 10^5 produced on detached young leaves (LPI -0.5) of A.Fi hybrid

TREATMENT	MEASURE	INCUBATION PERIOD (DAYS) 0	1	2	4	5	6	7	14	21
S. roseus alone										
Exp. 1	Mean	2.49	4.66	8.86	12.18	22.21	46.22	32.36	41.21	71.48
	S.D.	1.48	2.42	3.45	4.67	24.61	31.34	20.49	36.51	49.75
Exp. 2	Mean	1.76	2.49	5.04	3.53	16.72	126.85	33.84	44.77	66.10
	S.D.	1.37	2.32	4.23	3.17	14.33	53.47	22.69	24.32	38.39
Exp. 3	Mean	2.74	8.88	2.87	9.75	14.69	30.63	41.24	51.36	69.23
	S.D.	1.56	3.18	3.62	5.06	4.65	53.47	22.69	24.32	26.02
S. roseus + C. cladosporioides										
Exp. 1	Mean	2.41	2.80	3.66	5.29	7.78	11.47	23.85	31.72	27.7
	S.D.	1.70	1.78	2.49	2.96	2.35	3.31	15.88	25.55	25.27
Exp. 2	Mean	1.68	1.78	4.90	4.81	8.61	9.06	10.26	20.79	15.70
	S.D.	1.08	1.93	3.43	2.59	3.67	2.81	23.69	12.9	16.43
Exp. 3	Mean	2.51	6.94	3.37	7.11	8.51	7.76	17.57	37.90	28.88
	S.D.	2.00	2.79	3.31	2.93	3.05	2.22	15.96	30.1	27.09

C. cladosporioides: Spore germination on detached older leaves (LPI 1.5) of A. Nanum
(a) on leaf lamina

TREATMENT	MEASURE	INCUBATION PERIOD (DAYS) 0	1	2	4	5	6	7	14	21
C. cladosporioides alone	Mean	0	32.3	43.5	51.4	56.2	48.5	45.1	38.2	27.4
Exp. 1	S.D.		4.35	9.19	8.02	6.44	5.14	7.48	9.86	3.86
Exp. 2	Mean	0	28.3	37.6	45.4	59.7	49.8	51.2	43.1	31.4
	S.D.		6.80	5.28	9.21	5.23	6.41	9.16	7.55	6.61
Exp. 3	Mean	0	35.6	48.2	53.4	60.9	52.3	46.5	37.4	23.7
	S.D.		11.67	9.6	8.54	6.97	8.05	6.13	11.58	3.98
C. cladosporioides + S. roseus	Mean	0	26.9	32.8	37.5	48.7	56.3	59.7	48.4	29.8
Exp. 1	S.D.		5.18	6.88	9.22	7.88	7.33	4.28	5.22	5.61
Exp. 2	Mean	0	21.4	28.7	41.6	42.5	43.7	56.9	49.7	38.7
	S.D.		4.61	6.21	6.58	5.67	8.22	12.00	13.77	9.4
Exp. 3	Mean	0	30.7	3.75	36.5	48.9	56.3	64.4	51.3	25.0
	S.D.		7.34	3.33	3.64	6.59	5.28	4.37	7.71	5.57

S.D. = standard deviation on transformed data.

C. cladosporioides: Spore germination on detached older leaves (LPI 1.5) of A. Nanum (b) on leaf midrib

TREATMENT	MEASURE	INCUBATION PERIOD (DAYS) 0	1	2	4	5	6	7	14	21
C. cladosporioides alone Exp. 1	Mean	0	58.7	64.6	66.0	55.8	52.8	47.4	39.5	31.4
	S.D.		5.46	5.33	3.50	6.64	5.13	5.82	8.86	7.19
Exp. 2	Mean	0	53.7	59.9	68.2	59.2	56.2	48.2	37.9	26.4
	S.D.		4.67	4.04	6.92	5.66	5.89	8.20	10.22	9.63
Exp. 3	Mean	0	60.0	64.3	65.4	53.7	50.2	48.5	40.9	32.2
	S.D.		2.78	5.04	5.42	6.47	5.89	9.14	6.05	4.22
C. cladosporioides + S. roseus Exp. 1	Mean	0	45.3	53.1	54.8	55.4	61.9	56.2	49.2	40.3
	S.D.		5.45	5.77	7.83	6.68	4.22	6.63	5.89	11.38
Exp. 2	Mean	0	39.7	48.2	51.4	59.0	67.8	58.6	47.7	37.6
	S.D.		2.61	5.79	6.20	9.21	7.96	6.64	4.53	5.30
Exp. 3	Mean	0	49.3	51.0	51.5	55.8	62.5	63.9	64.1	39.4
	S.D.		2.34	3.05	1.71	4.18	5.37	6.40	9.49	2.66

S.D. = standard deviation on transformed data.

C. cladosporioides: Spore germination on detached older leaves (LPI 1.5) of A. Fi hybrid
(a) on leaf lamina

TREATMENT	MEASURE	INCUBATION PERIOD (DAYS) 0	1	2	4	5	6	7	14	21
C. cladosporioides alone										
Exp. 1	Mean	0	29.7	38.4	43.7	59.8	57.3	47.8	30.4	24.3
	S.D.		4.60	2.71	6.76	5.60	6.54	6.34	7.57	7.61
Exp. 2	Mean	0	31.1	34.4	31.2	43.6	57.2	55.1	27.5	13.9
	S.D.		4.17	4.89	5.54	5.25	5.15	6.63	6.31	8.05
Exp. 3	Mean	0	15.8	22.8	45.1	53.2	57.8	54.5	30.4	13.1
	S.D.		1.53	13.9	3.97	5.38	5.86	2.81	12.66	4.02
C. cladosporioides + S. roseus										
Exp. 1	Mean	0	15.2	24.6	39.8	50.2	52.1	48.6	42.7	39.6
	S.D.		3.84	6.63	4.74	8.47	5.70	6.23	4.93	5.41
Exp. 2	Mean	0	6.8	23.6	36.3	42.7	53.8	40.1	23.4	27.7
	S.D.		2.91	4.73	6.92	6.48	7.40	5.48	6.27	6.04
Exp. 3	Mean	0	9.5	18.4	45.8	46.4	51.1	53.3	36.2	18.4
	S.D.		3.5	5.77	5.42	6.06	6.38	7.31	3.95	2.83

S.D. = standard deviation on transformed data.

C. cladosporioides: Spore germination on detached older leaves (LPI 1.5) of A. Fi hybrid (b) on leaf midrib

TREATMENT	MEASURE	INCUBATION PERIOD (DAYS) 0	1	2	4	5	6	7	14	21
C. cladosporioides alone										
Exp. 1	Mean	0	38.9	44.7	55.2	60.3	51.3	42.9	33.4	21.0
	S.D.		4.06	4.9	4.21	7.41	6.48	4.34	6.51	6.18
Exp. 2	Mean	0	28.3	33.5	51.5	59.7	52.1	51.4	29.6	12.3
	S.D.		4.31	4.05	5.09	5.83	4.91	7.67	6.09	5.71
Exp. 3	Mean	0	18.9	26.3	55.7	59.5	57.3	55.2	38.5	27.9
	S.D.		1.8	20.35	9.39	5.89	6.87	9.11	10.23	11.06
C. cladosporioides + S. roseus										
Exp. 1	Mean	0	18.6	21.4	29.7	45.3	59.7	51.4	48.9	36.3
	S.D.		4.63	5.28	5.83	6.18	4.97	6.95	4.35	7.00
Exp. 2	Mean	0	12.4	22.3	23.3	39.9	57.2	58.2	40.5	32.4
	S.D.		2.73	7.93	6.46	5.72	4.06	6.54	5.70	6.9
Exp. 3	Mean	0	16.6	29.2	43.2	48.7	54.0	58.6	44.7	31.1
	S.D.		7.74	3.58	7.2	6.14	6.63	1.52	8.82	10.27

S.D. = standard deviation on transformed data.

C. cladosporioides: Spore germination on detached young leaves (LPI -0.5) of A. Narcan
(a) on leaf lamina

TREATMENT	MEASURE	INCUBATION PERIOD (DAYS) 0	1	2	4	5	6	7	14	21
C. cladosporioides alone	Mean	0	28.8	32.4	34.2	38.2	28.9	41.2	37.4	34.6
Exp. 1	S.D.		3.79	4.99	6.3	5.16	5.42	5.7	6.22	5.14
Exp. 2	Mean	0	31.4	34.6	39.7	42.7	39.3	43.4	45.2	39.7
	S.D.		4.96	4.02	5.33	5.41	5.79	6.28	6.66	9.34
Exp. 3	Mean	0	32.9	20.5	35.6	33.6	38.2	31.0	36.4	37.5
	S.D.		2.46	5.33	4.82	9.89	6.58	195.2	6.92	9.73
C. cladosporioides + S. roseus	Mean	0	16.3	24.6	28.4	27.6	27.9	30.2	31.3	29.9
Exp. 1	S.D.		4.17	5.34	6.61	6.08	5.95	6.54	9.2	5.33
Exp. 2	Mean	0	18.3	26.3	31.2	37.5	36.3	38.9	37.4	37.0
	S.D.		4.71	5.38	5.26	7.00	9.65	6.90	11.99	9.02
Exp. 3	Mean	0	19.7	20.2	30.1	34.5	25.9	21.8	31.0	32.8
	S.D.		3.45	7.05	5.79	8.52	4.32	3.13	5.33	6.9

S.D. = standard deviation on transformed data.

C. cladosporioides: Spore germination on detached young leaves (LPI -0.5) of A. Nanum (b) on leaf midrib

TREATMENT	MEASURE	INCUBATION PERIOD (DAYS) 0	1	2	4	5	6	7	14	21
C. cladosporioides alone										
Exp. 1	Mean	0	29.6	33.4	36.8	45.2	39.9	41.3	36.2	38.9
	S.D.		4.33	5.04	5.12	5.52	6.88	6.18	9.56	6.44
Exp. 2	Mean	0	32.6	36.9	42.4	44.9	43.5	46.9	44.8	48.2
	S.D.		4.41	3.55	7.06	6.22	6.46	4.75	4.29	9.79
Exp. 3	Mean	0	30.4	32.4	48.9	31.5	22.2	32.1	37.5	38.4
	S.D.		1.83	4.23	0.76	3.92	4.73	3.17	5.07	6.61
C. cladosporioides + S. roseus	Mean	0	17.4	28.2	29.6	33.1	34.7	32.6	31.7	30.0
Exp. 1	S.D.		3.44	5.34	4.35	6.91	4.74	9.11	6.27	6.49
Exp. 2	Mean	0	20.1	27.3	33.2	34.7	36.4	35.6	37.2	34.3
	S.D.		4.23	4.75	9.2	7.2	6.91	5.73	3.99	7.97
Exp. 3	Mean	0	18.2	34.6	42.9	33.4	23.0	28.8	33.4	36.2
	S.D.		4.23	5.21	5.82	3.22	3.5	3.97	4.74	6.15

S.D. = standard deviation on transformed data.

C. cladosporioides: Spore germination on young detached leaves (LPI -0.5) of A. Fi hybrid (a) on leaf lamina

TREATMENT	MEASURE	INCUBATION PERIOD 0	1	2	4	5	6	7	14	21
C. cladosporioides alone										
Exp. 1	Mean	0	32.4	36.2	39.2	43.5	40.3	38.7	39.6	40.3
	S.D.		4.23	5.38	9.19	4.20	4.98	9.08	5.90	7.25
Exp. 2	Mean	0	29.3	23.4	38.9	37.6	39.2	38.5	37.4	38.96
	S.D.		4.93	6.32	6.54	9.22	11.34	6.77	9.19	5.58
Exp. 3	Mean	0	34.9	38.8	41.8	37.8	40.3	43.2	46.7	43.0
	S.D.		24.18	21.8	15.75	10.12	9.36	11.99	4.95	9.19
C. cladosporioides + S. roseus										
Exp. 1	Mean	0	17.4	24.4	31.5	38.4	37.6	34.3	38.9	33.1
	S.D.		4.27	6.91	10.29	9.48	6.54	9.08	7.59	5.99
Exp. 2	Mean	0	16.2	25.5	29.8	33.4	38.2	34.3	36.2	37.5
	S.D.		4.3	9.34	3.79	4.75	9.9	4.97	6.78	8.76
Exp. 3	Mean	0	27.6	46.6	31.5	36.4	38.4	45.8	43.4	31.7
	S.D.		9.48	11.37	15.41	11.31	9.19	12.54	10.05	9.99

S.D. = standard deviation on transformed data

C. cladosporioides: Spore germination on detached young leaves (LPI -0.5) of A. Fi hybrid (b) on leaf midrib

TREATMENT	MEASURE	INCUBATION PERIOD (DAYS) 0	1	2	4	5	6	7	14	21
C. cladosporioides alone	Mean	0	33.4	38.7	43.6	45.9	48.7	43.2	46.4	44.6
Exp. 1	S.D.		4.44	2.59	6.28	6.88	4.16	5.38	9.09	7.03
Exp. 2	Mean	0	32.4	39.1	41.3	38.9	40.4	42.3	37.2	39.3
	S.D.		4.23	3.79	5.34	5.71	8.22	6.99	9.19	13.23
Exp. 3	Mean	0	24.4	37.6	42.1	43.8	39.7	43.1	50.4	42.9
	S.D.		20.79	16.48	11.38	8.82	15.59	13.87	9.48	8.82
C. cladosporioides + S. roseus	Mean	0	21.3	28.4	35.7	35.8	32.5	38.8	39.4	37.6
Exp. 1	S.D.		5.33	4.73	6.21	4.03	6.73	4.87	5.65	6.52
Exp. 2	Mean	0	19.2	28.4	27.2	33.1	37.2	33.4	36.4	37.9
	S.D.		4.22	5.34	8.6	6.53	5.77	16.86	6.96	7.65
Exp. 3	Mean	0	26.4	21.5	49.4	38.7	39.0	43.7	43.7	36.1
	S.D.		6.61	11.16	9.38	9.21	11.20	9.19	14.73	9.99

S.D. = standard deviation on transformed data

C. cladosporioides: Spore germ tube growth (μm) on detached older leaves (LPI 1.5) of A. Nanum (a) on leaf lamina

TREATMENT	MEASURE	INCUBATION PERIOD (DAYS) 0	1	2	4	5	6	7	14	21
C. cladosporioides alone	Mean	0	15.5	18.8	27.9	29.4	31.2	32.6	82.3	227.4
Exp. 1	S.D.		3.76	1.44	7.92	11.13	6.59	13.36	20.99	49.14
Exp. 2	Mean	0	14.4	18.3	26.1	27.8	29.9	32.6	82.3	227.4
	S.D.		4.27	3.34	6.84	7.25	6.65	1.44	13.42	37.53
Exp. 3	Mean	0	12.8	18.4	26.6	28.4	29.3	31.5	78.6	215.4
	S.D.		4.04	3.43	19.0	7.95	9.22	6.90	18.25	56.92
C. cladosporioides + S. roseus	Mean	0	10.9	14.7	18.1	21.7	27.9	29.6	50.6	153.6
Exp. 1	S.D.		3.5	1.75	2.23	5.31	9.23	3.82	25.44	36.57
Exp. 2	Mean	0	9.7	15.7	14.7	25.2	26.7	29.0	59.5	137.4
	S.D.		3.92	3.03	13.3	12.09	7.97	16.03	17.05	30.06
Exp. 3	Mean	0	9.2	9.3	15.9	24.2	25.1	25.5	42.2	133.2
	S.D.		2.5	2.0	1.9	5.2	3.7	3.2	9.6	2.6

C. cladosporioides: Spore germ tube growth (μm) on detached older leaves (LPI 1.5) of A. Nanum (b) on leaf midrib

TREATMENT	MEASURE	INCUBATION PERIOD (DAYS) 0	1	2	4	5	6	7	14	21
C. cladosporioides alone	Mean	0	25.3	24.9	27.3	30.6	29.9	37.4	94.8	243.1
Exp. 1	S.D.		4.96	3.03	5.31	12.41	9.86	9.16	15.59	37.85
Exp. 2	Mean	0	24.8	26.8	29.3	27.3	34.1	39.7	106.2	282.7
	S.D.		5.06	1.21	11.13	6.9	9.99	13.26	18.32	43.57
Exp. 3	Mean	0	24.4	24.1	36.3	27.4	30.9	34.9	70.9	232.8
	S.D.		5.10	5.90	21.90	13.2	11.13	14.6	17.1	47.7
C. cladosporioides + S. roseus	Mean	0	15.8	17.2	18.8	20.1	23.6	30.3	81.0	166.6
Exp. 1	S.D.		5.09	3.82	4.46	8.24	9.23	13.93	21.69	30.09
Exp. 2	Mean	0	14.4	16.4	20.8	19.4	22.9	30.6	85.2	199.5
	S.D.		3.82	21.82	9.10	7.54	8.69	9.13	18.38	30.60
Exp. 3	Mean	0	15.0	13.2	15.3	17.4	24.6	26.2	52.7	190.6
	S.D.		3.7	1.2	0.85	4.66	10.15	4.29	27.98	27.35

C. cladosporioides: Spore germ tube growth (µm) on detached older leaves (LPI 1.5) of A. Fi hybrid (a) on leaf lamina

TREATMENT	MEASURE	INCUBATION PERIOD (DAYS) 0	1	2	4	5	6	7	14	21
C. cladosporioides alone										
Exp. 1	Mean	0	13.9	18.5	20.5	20.7	20.1	26.2	69.0	90.3
	S.D.		4.23	7.54	1.44	10.31	8.88	9.04	25.13	28.15
Exp. 2	Mean	0	15.4	18.6	29.6	36.3	41.3	45.6	80.1	93.5
	S.D.		4.39	7.41	14.95	12.25	8.91	12.41	29.99	35.94
Exp. 3	Mean	0	14.5	17.3	16.9	19.9	24.0	28.9	73.1	81.7
	S.D.		0.64	0.39	1.78	3.79	4.3	4.39	37.68	19.18
C. cladosporioides + S. roseus										
Exp. 1	Mean	0	9.6	15.1	17.9	18.6	19.7	25.3	62.9	68.1
	S.D.		2.48	3.79	3.92	5.86	6.52	8.57	24.39	39.91
Exp. 2	Mean	0	12.3	15.5	18.8	26.8	32.7	46.4	84.3	102.1
	S.D.		1.07	2.2	5.38	6.52	8.24	6.17	31.36	38.07
Exp. 3	Mean	0	8.8	15.3	17.4	19.7	23.5	27.9	46.1	52.5
	S.D.		0.83	28.0	2.0	5.03	4.39	4.84	3.76	2.87

C. cladosporioides: Spore germ tube growth (μm) on detached older leaves (LPI 1.5) of A. Fi hybrid (b) on leaf midrib

TREATMENT	MEASURE	INCUBATION PERIOD (DAYS) 0	1	2	4	5	6	7	14	21
C. cladosporioides alone										
Exp. 1	Mean	0	17.4	18.9	24.3	30.1	31.1	33.5	68.7	112.7
	S.D.		1.75	4.46	5.38	7.64	9.1	7.95	11.01	49.17
Exp. 2	Mean	0	17.1	16.4	24.6	34.9	39.9	46.3	89.5	123.1
	S.D.		4.58	2.87	6.94	7.32	12.72	21.21	9.86	45.4
Exp. 3	Mean	0	16.1	20.4	24.6	27.4	29.9	34.8	73.1	108.4
	S.D.		1.28	3.76	5.0	3.94	7.32	7.86	24.68	55.34
C. cladosporioides + S. roseus	Mean	0	12.5	14.7	16.7	26.4	27.3	31.2	52.5	83.6
Exp. 1	S.D.		2.68	3.79	4.46	5.09	7.6	9.64	15.81	37.94
Exp. 2	Mean	0	13.1	17.6	14.7	26.9	30.6	42.6	78.4	113.3
	S.D.		1.05	2.45	3.82	3.63	6.52	8.4	28.27	43.89
Exp. 3	Mean	0	12.2	15.6	16.5	23.8	28.1	30.7	45.0	63.6
	S.D.		1.21	3.25	2.23	5.47	7.41	5.38	10.5	11.26

C. cladosporioides: Spore germ tube growth (µm) on detached young leaves (LPI -0.5) of A. Nanum (a) on leaf lamina

TREATMENT	MEASURE	INCUBATION PERIOD (DAYS) 0	1	2	4	5	6	7	14	21
C. cladosporioides alone										
Exp. 1	Mean	0	15.8	16.6	18.7	20.1	18.2	22.2	36.4	40.9
	S.D.		3.12	4.65	6.81	5.09	9.35	5.41	13.68	6.68
Exp. 2	Mean	0	13.5	18.9	17.3	22.1	16.9	22.9	34.9	39.8
	S.D.		0.77	8.65	3.12	4.55	6.27	8.72	7.03	10.24
Exp. 3	Mean	0	19.5	31.3	29.0	30.6	28.9	21.9	39.8	44.2
	S.D.		4.01	4.39	2.87	5.7	2.74	2.23	6.05	10.82
C. cladosporioides + S. roseus	Mean	0	11.8	15.8	16.9	19.0	19.9	17.8	33.1	36.6
Exp. 1	S.D.		1.34	5.73	4.17	5.47	6.81	2.48	6.33	10.21
Exp. 2	Mean	0	13.5	15.3	15.8	16.5	18.6	18.2	29.4	34.5
	S.D.		1.02	2.29	6.01	5.44	4.77	7.73	5.12	12.63
Exp. 3	Mean	0	11.0	28.2	23.9	25.3	29.9	19.8	36.4	40.8
	S.D.		3.92	2.55	5.28	5.73	2.2	0.81	5.95	7.73

C. cladosporioides: Spore germ tube growth (μm) on detached young leaves (LPI -0.5) of A. Nanum
(b) on leaf midrib

TREATMENT	MEASURE	0	1	2	4	5	6	7	14	21
C. cladosporioides alone										
Exp. 1	Mean	0	16.9	18.2	20.7	18.8	21.9	23.2	39.4	43.0
	S.D.		1.34	2.29	6.17	8.75	4.52	5.12	10.82	9.45
Exp. 2	Mean	0	15.54	17.9	16.6	22.6	17.3	25.3	36.9	40.4
	S.D.		1.82	9.04	4.2	5.06	8.65	5.82	8.94	11.52
Exp. 3	Mean	0	22.2	34.5	35.1	31.5	38.1	36.4	40.4	45.2
	S.D.		1.78	6.05	6.55	3.06	1.44	2.36	8.59	10.5
C. cladosporioides + S. roseus										
Exp. 1	Mean	0	15.8	17.8	18.7	18.3	19.0	22.9	32.5	38.1
	S.D.		2.77	7.70	4.82	4.58	9.48	2.71	5.19	12.63
Exp. 2	Mean	0	10.5	15.5	16.9	18.6	18.8	21.8	29.8	33.7
	S.D.		2.29	2.68	3.85	4.9	5.7	4.27	6.01	10.18
Exp. 3	Mean	0	10.5	17.8	18.2	26.5	33.3	27.3	36.0	42.6
	S.D.		3.15	3.37	4.27	6.81	2.71	3.47	6.11	8.62

C.cladosporioides: Spore germ tube growth (µm) on detached young leaves (LPI -0.5) of A. Fi hybrid (a) on leaf lamina

TREATMENT	MEASURE	INCUBATION PERIOD (DAYS) 0	1	2	4	5	6	7	14	21
C. cladosporioides alone	Mean	0	13.8	21.9	17.7	21.9	22.9	23.7	39.4	43.8
Exp. 1	S.D.		1.82	5.73	11.45	2.68	5.09	1.91	8.91	12.72
Exp. 2	Mean	0	17.4	19.7	19.6	23.7	25.4	22.9	41.3	46.4
	S.D.		2.45	4.49	5.73	2.93	6.05	7.95	18.45	25.13
Exp. 3	Mean	0	8.4	7.6	10.5	16.5	20.5	32.1	10.2	21.6
	S.D.		3.31	1.72	3.25	1.98	3.63	29.39	2.23	2.10
C. cladosporioides + S. roseus	Mean	0	14.0	14.9	27.5	21.3	21.9	33.9	37.1	45.8
Exp. 1	S.D.		1.15	1.34	10.02	2.64	7.64	7.13	9.86	12.6
Exp. 2	Mean	0	16.9	16.8	20.1	22.9	20.8	22.2	35.9	41.0
	S.D.		4.84	2.17	6.27	7.76	4.49	7.38	23.6	16.22
Exp. 3	Mean	0	6.8	9.1	9.9	15.4	18.9	23.4	24.7	22.9
	S.D.		0.7	2.99	0.29	6.3	7.86	8.59	16.79	6.46

C. cladosporioides: Spore germ tube growth (μm) on detached young leaves (LPI -0.5) of A. Fi hybrid (b) on leaf midrib

TREATMENT	MEASURE	INCUBATION PERIOD (DAYS) 0	1	2	4	5	6	7	14	21
C. cladosporioides alone	Mean	0	13.8	20.1	26.1	25.2	27.0	23.4	33.7	43.3
Exp. 1	S.D.		1.13	2.39	2.29	5.73	7.0	4.62	5.41	14.22
Exp. 2	Mean	0	11.9	23.3	26.1	28.5	23.9	23.7	44.2	50.8
	S.D.		1.66	3.06	7.73	4.04	8.27	5.86	15.27	16.86
Exp. 3	Mean	0	12.8	8.6	15.1	21.9	24.9	29.5	10.6	25.4
	S.D.		25.2	1.91	4.96	2.74	9.23	13.74	3.72	6.97
C. cladosporioides + S. roseus	Mean	0	17.7	18.1	29.3	23.5	26.4	27.2	33.5	51.4
Exp. 1	S.D.		3.34	2.07	2.48	4.49	6.17	1.34	2.99	18.13
Exp. 2	Mean	0	17.5	21.9	23.3	23.9	24.2	26.2	40.8	42.4
	S.D		3.12	4.58	5.16	2.48	5.06	7.83	26.4	20.1
Exp. 3	Mean	0	13.4	12.3	13.5	18.4	21.6	22.4	23.8	30.5
	S.D.		2.68	4.2	1.59	9.45	10.88	7.28	15.14	7.51

Key of symbols used in tables of data on microbial interactions

Data on:-

1) P. antirrhini

P = P. antirrhini alone

PC = P. antirrhini + C. cladosporioides

PS = P. antirrhini + S. roseus

PCS = P. antirrhini + C. cladosporioides + S. roseus

2) C. cladosporioides

C = C. cladosporioides alone

CP = C. cladosporioides + P. antirrhini

CS = C. cladosporioides + S. roseus

CPS = C. cladosporioides + P. antirrhini + S. roseus

3) S. roseus

S = S. roseus alone

SP = S. roseus + P. antirrhini

SC = S. roseus + C. cladosporioides

SPC = S. roseus + P. antirrhini + C. cladosporioides

S.D. = Standard deviation on transformed data

Standard Deviation = Standard deviation on raw data

Min = Minimum

Max = Maximum

25% Quart = 25% Quartile

75% Quart = 75% Quartile

Interactions in vitro: Experiment 1

P. antirrhini: % spore germination

	P	PC	PS	PCS
Mean	64.69	59.65	38.4	69.82
S.D.	9.98	6.17	6.6	5.88

P. antirrhini: germ tube length (μm)

	P	PC	PS	PCS
Median	272.7	361.15	153.68	372.67
Min.	73.3	171.1	24.4	97.8
Max.	879.8	586.6	464.4	672.0
25% Quart	183.3	287.2	97.8	281.1
75% Quart	384.9	427.7	232.2	433.8

C. cladosporioides: % spore germination

	C	CP	CS	CPS
Mean	60.3	47.1	44.1	54.7
S.D.	3.98	0.79	6.36	3.1

C. cladosporioides: germ tube length (μm)

	C	CP	CS	CPS
Median	22.3	19.1	15.9	15.9
Min.	6.4	6.4	6.4	6.4
Max.	92.2	114.5	92.2	101.8
25% Quart	15.9	15.9	9.54	12.7
75% Quart	34.9	31.8	22.3	31.8

S. roseus: total volume of cells (μm^3)x 10^6

	S	SP	SC	SPC
Mean	1.85	5.24	4.3	3.85
Standard Deviation	0.26	0.53	0.48	0.4

Interactions in vitro: Experiment 2

P. antirrhini: % spore germination

	P	PC	PS	PCS
Mean	84.2	76.6	52.3	81.3
S.D.	8.4	9.3	12.2	7.1

P. antirrhini: germ tube length (μm)

	P	PC	PS	PCS
Median	287.2	384.9	177.2	409.3
Min.	69.3	109.9	36.7	73.3
Max.	847.3	476.6	305.5	513.2
25% Quart	148.4	305.5	110.0	256.6
75% Quart	405.7	439.9	293.3	452.1

C. cladosporioides: % spore germination

	C	CP	CS	CPS
Mean	82.4	67.9	73.4	75.3
S.D.	4.5	2.5	7.9	6.7

C. cladosporioides: germ tube length (μm)

	C	CP	CS	CPS
Median	27.0	22.3	17.5	20.67
Min.	6.4	3.2	6.4	3.2
Max.	84.3	76.3	95.6	111.3
25% Quart	12.7	9.5	9.5	12.7
75% Quart	38.2	35.0	22.3	25.4

S. roseus: total volume of cells (μm^3) x 10^6

	S	SP	SC	SPC
Mean	0.85	3.13	1.84	2.24
Standard Deviation	0.2	0.67	0.5	0.34

Interactions on detached leaves: P. antirrhini. 2 day test
Experiment 1

		% spore germination			
CULTIVAR	MEASURE	P	PC	PS	PCS
A. Nanum	Mean	81.2	68.1	74.74	74.3
	S.D.	8.55	8.90	6.79	9.48
A. Fi hybrid	Mean	51.9	70.5	81.2	77.34
	S.D.	1.7	5.3	7.37	13.6

		Germ tube lengths (µm)			
		P	PC	PS	PCS
A. Nanum	Median	248.0	174.9	268.7	337.1
	Min.	12.7	15.9	120.8	95.4
	Max.	397.5	492.9	588.3	670.9
	25% Quart	47.7	95.4	209.9	276.6
	75% Quart	302.1	349.8	588.3	451.6
A. Fi hybrid	Median	54.1	63.6	416.6	340.3
	Min.	6.36	15.9	232.1	95.4
	Max.	321.18	232.2	667.8	705.9
	25% Quart	25.4	38.2	325.9	197.2
	75% Quart	90.6	130.4	488.1	470.6

		% leaf penetration			
		P	PC	PS	PCS
A. Nanum	Mean	41.3	23.8	27.0	20.6
	S.D	14.82	8.79	5.48	4.22
A. Fi hybrid	Mean	6.6	1.6	28.4	20.3
	S.D.	3.22	4.73	3.15	11.21

Interactions on detached leaves: P. antirrhini. 2 day test
Experiment 2

% spore germination

CULTIVAR	MEASURE	P	PC	PS	PCS
A. Nanum	Mean	89.3	70.0	78.6	74.3
	S.D.	7.6	8.4	7.9	8.5
A. Fi hybrid	Mean	54.8	67.3	74.3	75.7
	S.D.	2.4	5.3	8.1	9.3

Germ tube lengths (µm)

		P	PC	PS	PCS
A. Nanum	Median	225.8	152.6	251.2	298.9
	Min.	22.3	15.9	117.7	89.0
	Max.	375.2	411.8	610.6	729.2
	25% Quart	50.9	76.3	173.3	232.1
	75% Quart	311.6	335.5	511.9	399.4
A. Fi hybrid	Median	57.2	60.4	365.7	289.4
	Min.	15.9	12.7	207.7	76.3
	Max.	365.7	220.4	594.7	626.5
	25% Quart	22.3	47.7	310.1	171.7
	75% Quart	98.6	117.7	453.2	411.8

% leaf penetration

		P	PC	PS	PCS
A. Nanum	Mean	43.4	27.2	28.9	20.0
	S.D.	12.5	12.4	7.2	9.6
A. Fi hybrid	Mean	5.8	1.2	24.3	23.8
	S.D.	3.6	2.1	5.7	5.9

Interactions on detached leaves : P. antirrhini. 6 day test
Experiment 1

% spore germination

CULTIVAR	MEASURE	P	PC	PS	PCS
A. Nanum	Mean	64.6	64.2	76.2	35.8
	S.D.	6.06	3.43	5.8	2.1
A. Fi hybrid	Mean	58.3	36.8	67.6	39.8
	S.D.	2.33	17.52	5.86	8.15

Germ tube lengths (µm)

		P	PC	PS	PCS
A. Nanum	Median	63.6	47.7	133.56	127.2
	Min.	15.9	15.9	47.7	15.9
	Max.	257.6	222.6	298.9	270.3
	25% Quart	31.8	31.8	101.8	47.7
	75% Quart	101.8	69.9	192.4	166.9
A. Fi hybrid	Median	120.84	31.8	79.5	47.7
	Min.	31.8	9.5	15.9	15.9
	Max.	349.8	318.0	232.1	130.4
	25% Quart	69.9	22.3	42.9	31.8
	75% Quart	187.6	186.0	120.8	66.8

% leaf penetration

		P	PC	PS	PCS
A. Nanum	Mean	39.1	19.0	21.1	1.0
	S.D.	4.12	4.1	1.01	0.4
A. Fi hybrid	Mean	18.1	0	12.4	13.8
	S.D.	0.85		4.74	6.73

Interactions on detached leaves: P. antirrhini. 6 day test
Experiment 2

% spore germination

CULTIVAR	MEASURE	P	PC	PS	PCS
A. Nanum	Mean	68.5	67.4	77.5	40.4
	S.D.	7.4	5.8	6.2	3.8
A. Fi hybrid	Mean	60.9	38.7	68.3	45.4
	S.D.	4.7	8.9	7.3	9.2

Germ tube lengths (µm)

		P	PC	PS	PCS
A. Nanum	Median	79.5	54.06	143.1	124.0
	Min.	19.1	12.7	54.1	19.1
	Max.	380.0	235.3	318.0	295.7
	25% Quart	38.2	19.1	85.9	82.7
	75% Quart	114.5	76.3	235.3	149.5
A. Fi hybrid	Median	114.5	36.6	82.9	44.5
	Min.	34.9	6.4	19.1	12.7
	Max.	308.5	283.0	283.0	117.7
	25% Quart	19.1	20.7	47.7	34.9
	75% Quart	165.4	135.2	133.7	76.3

% leaf penetration

	P	PC	PC	PS	PCS
A. Nanum	Mean	42.2	17.6	23.4	2.5
	S.D.	5.6	6.8	5.7	1.4
A. Fi hybrid	Mean	21.3	0	13.5	15.6
	S.D.	1.3		7.4	6.2

Interactions on detached leaves: C. cladosporioides on A. Nanum
2 day test. Experiment 1

POSITION ON LEAF	MEASURE	% spore germination C	CP	CS	CPS
Lamina	Mean	73.4	56.12	69.99	69.8
	S.D.	9.58	14.97	5.48	5.32
Midrib	Mean	84.9	70.44	79.57	71.49
	S.D.	4.64	6.37	1.75	0.27

		Germ tube length (µm) C	CP	CS	CPS
Lamina	Median	25.4	28.62	22.26	15.9
	Min.	6.4	6.4	6.4	6.4
	Max.	63.6	127.2	79.5	41.3
	25% Quart	15.9	15.9	15.9	15.9
	75% Quart	31.8	47.7	31.8	22.3
Midrib	Median	57.2	31.8	25.44	25.40
	Min.	9.5	12.7	9.5	15.9
	Max.	127.2	79.5	82.7	69.9
	25% Quart	31.8	15.9	15.9	15.9
	75% Quart	79.5	47.7	38.2	31.8

Interactions on detached leaves: C. cladosporioides on A. Nanum
2 day test. Experiment 2

POSITION ON LEAF	MEASURE	% spore germination C	CP	CS	CPS
Lamina	Mean	68.2	52.4	58.7	56.3
	S.D.	5.3	10.9	6.4	5.7
Midrib	Mean	79.8	58.9	64.5	61.0
	S.D.	8.1	12.5	8.4	4.7

		Germ tube lengths (µm) C	CP	CS	CPS
Lamina	Median	17.5	21.2	15.4	12.7
	Min.	6.4	3.2	3.2	9.5
	Max.	40.3	83.8	54.1	38.2
	25% Quart	9.5	12.7	9.5	9.5
	75% Quart	25.4	38.2	22.3	19.1
Midrib	Median	50.8	25.4	44.5	31.8
	Min.	12.7	6.4	9.5	7.4
	Max.	120.8	66.7	54.1	60.4
	25% Quart	38.1	12.7	15.9	17.8
	75% Quart	69.9	38.2	34.9	31.8

Interactions on detached leaves: C. cladosporioides on A. Fi hybrid
2 day test. Experiment 1

POSITION ON LEAF	MEASURE	% spore germination C	CP	CS	CPS
Lamina	Mean	67.58	72.68	62.13	55.49
	S.D.	3.2	1.64	5.15	0.35
Midrib	Mean	90.75	78.65	83.28	62.87
	S.D.	4.8	1.34	2.08	6.29

		Germ tube lengths (µm) C	CP	CS	CPS
Lamina	Median	31.8	27.0	15.9	17.4
	Min.	6.4	6.4	6.4	6.4
	Max.	197.2	95.4	31.8	47.7
	25% Quart	22.3	15.9	15.9	12.7
	75% Quart	47.7	50.9	28.6	22.3
Midrib	Median	73.14	44.52	31.8	20.67
	Min.	15.9	15.9	12.7	9.5
	Max.	200.3	79.5	66.8	47.7
	25% Quart	54.1	27.0	15.9	15.9
	75% Quart	101.8	63.6	38.2	31.8

Interactions on detached leaves: C. cladosporioides on A. Fi hybrid
2 day test. Experiment 2

POSITION ON LEAF	MEASURE	% spore germination C	CP	CS	CPS
Lamina	Mean	62.4	63.2	55.4	50.2
	S.D.	4.8	5.7	1.64	2.3
Midrib	Mean	77.3	66.1	69.4	57.3
	S.D.	3.3	5.9	4.8	5.7

		Germ tube lengths (µm) C	CP	CS	CPS
Lamina	Median	44.0	38.2	12.7	14.3
	Min.	12.7	6.4	3.2	9.5
	Max.	283.0	171.7	38.2	34.9
	25% Quart	31.8	9.5	15.9	12.7
	75% Quart	63.6	60.4	22.2	15.9
Midrib	Median	76.3	41.3	47.2	28.6
	Min.	19.1	15.9	9.5	3.2
	Max.	209.9	98.6	79.5	57.2
	25% Quart	44.5	22.3	25.4	19.1
	75% Quart	111.3	60.4	63.6	35.0

Interactions on detached leaves: C. cladosporioides on A. Nanum
6 day test. Experiment 1

POSITION ON LEAF	MEASURE	% spore germination C	CP	CS	CPS
Lamina	Mean	41.19	44.99	64.33	59.24
	S.D.	3.1	5.97	4.49	21.9
Midrib	Mean	46.87	62.0	66.2	68.87
	S.D.	3.2	2.9	4.6	7.6

		Germ tube lengths (µm) C	CP	CS	CPS
Lamina	Median	15.9	15.9	15.9	22.2
	Min.	6.4	6.4	6.4	9.5
	Max.	63.6	47.7	63.6	54.1
	25% Quart	15.9	15.9	15.9	15.9
	75% Quart	22.3	31.8	22.3	31.8
Midrib	Median	31.8	22.26	25.44	31.8
	Min.	9.5	6.4	6.4	9.5
	Max.	69.9	38.2	63.6	63.6
	25% Quart	22.3	15.9	15.9	25.4
	75% Quart	44.5	31.8	31.8	42.9

Interactions on detached leaves: C. cladosporioides on A. Nanum
6 day test. Experiment 2

POSITION ON LEAF	MEASURE	% spore germination C	CP	CS	CPS
Lamina	Mean	38.7	45.2	59.8	52.0
	S.D.	4.2	4.3	5.2	8.9
Midrib	Mean	42.3	58.9	61.4	58.7
	S.D.	4.9	11.2	4.9	10.8

		Germ tube lengths (µm) C	CP	CS	CPS
Lamina	Median	22.2	12.7	15.9	19.1
	Min.	6.4	3.2	3.2	9.5
	Max.	79.5	57.2	66.8	60.4
	25% Quart	19.1	6.4	12.7	15.9
	75% Quart	54.1	38.2	41.3	50.9
Midrib	Median	41.3	22.3	19.1	38.2
	Min.	15.9	3.2	9.5	12.7
	Max.	79.5	44.5	35.0	69.9
	25% Quart	25.4	12.7	19.9	28.6
	75% Quart	54.1	35.0	31.8	47.7

Interactions on detached leaves: C. cladosporioides on A. Fi hybrid
6 day test. Experiment 1

POSITION ON LEAF	MEASURE	% spore germination C	CP	CS	CPS
Lamina	Mean	50.80	56.0	52.30	41.54
	S.D.	2.65	3.78	4.75	0.38
Midrib	Mean	47.97	69.42	65.12	32.90
	S.D.	2.68	2.96	1.95	5.59

		Germ tube lengths (µm) C	CP	CS	CPS
Lamina	Median	28.62	15.9	17.4	31.8
	Min.	6.4	6.4	6.4	6.4
	Max.	66.8	54.1	63.6	133.6
	25% Quart	15.9	12.7	15.9	15.9
	75% Quart	31.8	19.1	22.3	57.2
Midrib	Median	31.8	31.8	31.8	38.16
	Min.	6.4	15.9	6.36	12.7
	Max.	95.4	76.3	69.9	139.9
	25% Quart	15.9	22.3	15.9	31.8
	75% Quart	47.7	31.8	38.2	79.5

Interactions on detached leaves: C. cladosporioides on A. Fi hybrid
6 day test. Experiment 2

POSITION ON LEAF	MEASURE	% spore germination C	CP	CS	CPS
Lamina	Mean	45.3	49.5	51.0	36.4
	S.D.	3.4	1.2	1.7	4.5
Midrib	Mean	43.3	62.8	59.7	29.7
	S.D.	3.7	2.4	1.5	5.2

		Germ tube lengths (µm) C	CP	CS	CPS
Lamina	Median	25.4	12.72	19.1	28.6
	Min.	3.2	3.2	6.4	9.54
	Max.	60.4	50.9	57.2	139.9
	25% Quart	15.9	9.5	12.7	19.1
	75% Quart	44.5	19.1	28.6	66.8
Midrib	Median	28.62	22.26	28.6	31.8
	Min.	19.1	6.4	12.7	15.9
	Max.	89.0	63.6	79.5	146.3
	25% Quart	25.4	15.9	15.9	35.0
	75% Quart	50.9	25.4	44.5	85.9

Interactions on detached leaves: S. roseus. Total volume of cells (μm^3) per leaf x 10^5

2 day test. Experiment 1

CULTIVAR	MEASURE	S	SP	SC	SPC
A. nanum	Mean	18.2	33.3	15.8	24.3
	Standard Deviation	0.64	1.68	0.53	1.36
A. fi hybrid	Mean	22.3	31.4	13.3	19.5
	Standard Deviation	0.29	1.03	0.53	1.10

6 day test. Experiment 1

A. nanum	Mean	1642	2202	13.9	128.6
	Standard Deviation	187	177.8	2.49	58.4
A. fi hybrid	Mean	1552.5	1470.6	17.2	431.4
	Standard Deviation	368.9	144.2	12.89	151.4

2 day test. Experiment 2

A. nanum	Mean	20.4	35.2	16.5	22.6
	Standard Deviation	0 .76	1.52	1.04	1.85
A. fi hybrid	Mean	18.9	34.2	14.2	17.4
	Standard Deviation	0 .84	1.43	0.96	1.32

6 day test. Experiment 2

A. nanum	Mean	1890	2432	15.7	142.3
	Standard Deviation	204	163.2	3.1	71.3
A. fi hybrid	Mean	1741	1683	21.5	547
	Standard Deviation	194.3	157.8	6.4	184.2

APPENDIX 2

Description of Antirrhinum majus L. Fi hybrid tall forcing strain orange scarlet.

The plant is an annual or bi-annual of erect habit growing to a height of 1 m in the open or 1.5 m under glass. Leaves are linear oblong (55-65 mm x 20-25 mm) with an entire margin. Flower buds are produced in both axillary and terminal positions to give a flowering spike up to 150 mm in length. The corolla, of length 60 mm, is orange scarlet in colour with a golden coloured palate.

Species of filamentous fungi isolated from needles of Norway spruce in 1971

(A) Trees free from Chrysomyxa abietis in the previous year

(B) Trees infected by Chrysomyxa abietis in the previous year

Species isolated	(A)	(B)
Acremonium sp.	-	+
Alternaria tenuis Nees.	+	-
Aspergillus brunneo-uniseriatus Singh & Bakshi	-	+
Aspergillus clavatus Desm.	-	+
Aspergillus fumigatus Fres.	+	+
Aspergillus janus Raper & Thom	+	+
Aspergillus nidulans Wint.	-	+
Aspergillus terreus Thom	+	-
Aspergillus wintii Wehmer	-	+
Aureobasidium pullulans (de Bary) Arn.	+	+
Bispora sp.	+	+
Botrytis cinerea Pers. ex Pers.	+	+
Botrytis pyrimidalis (Bonorden) Sacc.	-	+
Cephalosporium sp.	+	+
Chaetopsis sp.	+	-
Chalaropsis sp.	+	+
Cladosporium cladosporioides (Fresen.) de Vries	+	+
Cladosporium herbarum (Pers.) Link ex S.F. Gray	+	+
Dicoccum sp.	+	+
Diplodia sp.	-	+
Fusarium aquaeductuum Lagh.	+	-
Fusarium oxysporum Schlect.	+	+
Gonatobotrys sp.	+	-

	(A)	(B)
Helicoma sp.	+	-
Heptaster sp.	+	-
Hormiscium sp.	-	+
Humicola sp.	+	-
Hylodendron sp.	+	-
Leptosphaera sp.	+	-
Monilia sp.	+	+
Mucor genevensis Lend.	+	+
Mucor microsporus Nam.	+	-
Penicillium cyclopium West.	-	+
Penicillium fellutanum Biourge	-	+
Penicillium frequentans West.	+	+
Penicillium funiculosum Thom	+	+
Penicillium lapidosum Raper & Fennell	+	+
Penicillium thomi Zaleski	+	+
Periconia sp.	-	+
Periconiella sp.	+	+
Sclerotium sp.	+	-
Scopulariopsis sp.	+	-
Sympodiella sp.	+	-
Trichophyton sp.	+	-
Trichothecium sp.	-	+
Tritirachium sp.	+	+
Sterile white mycelia	+	+